University of Alberta

PHYSICAL ACTIVITY AND ESTROGEN METABOLISM IN PREMENOPAUSAL WOMEN: AN OBSERVATIONAL STUDY AND A RANDOMIZED CONTROLLED TRIAL.

by

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Abstract

The purpose of this dissertation was to examine the effects of physical activity on estrogen metabolism, a proposed biomarker of breast cancer risk, in premenopausal women.

Study One examined the association between physical activity and estrogen metabolites by comparing estrogen metabolite levels in women with high levels of aerobic fitness to women with average levels of aerobic fitness. Urinary estrogen metabolite levels, 2-OHE1, 16α-OHE1, and 2:16α-OHE1 in the follicular and luteal phase of the menstrual cycle of 17 women with high fitness (mean $VO_2max=52.8 \pm 0.9$ ml/kg/min) were compared to 13 women with average fitness (mean $VO_2max = 35.0 \pm 0.93$ ml/kg/min). Both groups were similar in age, height, body weight and body mass index (BMI). However, members of the high fitness group were leaner than the average fitness group. No statistically significant differences in 2-OHE1, 16α-OHE1, and 2:16α-OHE1 were seen between the groups. Further analysis showed that overall, a higher BMI was associated with lower follicular 2-OHE1 (r=-.37, p=.038) and lower follicular 2:16α-OHE1 (r=-.40, p=.026), and higher sum of four skinfolds (SSF) was associated with a higher luteal 16α-OHE1 level (r=.39, p=.032) and lower luteal phase 2:16α-OHE1 (r=-.41, p=.021), suggesting a lower breast cancer risk in women with lower BMI and lower SSF.

Study Two tested a 12-week aerobic exercise training intervention to examine the effects of exercise training on estrogen metabolites using randomized controlled trial methodology. The exercise intervention was designed to improve aerobic fitness and to

examine the effect of aerobic exercise training on estrogen metabolism in 32 premenopausal women. At baseline no difference in age, aerobic fitness, body composition, reproductive factors or estrogen metabolites levels was observed between the 17 participants assigned to the exercise group and the 15 participants assigned to the control group. Overall adherence to the exercise intervention was 91%. Despite a significant (14%) increase in aerobic fitness in the exercise group, no significant difference in luteal 2-OHE1, 16α-OHE1 or 2:16α-OHE1 was observed between the groups and change in aerobic fitness was not associated with a change in estrogen metabolites. However, at baseline an inverse association between 2:16α-OHE1 and percent body fat (r=-.40, p=.044) was seen for all participants, and a change in lean body mass was positively associated with change 2:16α-OHE1 (r=.53, p=.029), again suggesting that body composition may be associated with estrogen metabolism.

In summary, this research suggests that aerobic fitness may not be associated with changes in estrogen metabolism. No difference in 2-OHE1, 16α-OHE1 or 2:16α-OHE1 was observed in high aerobic fitness and average aerobic fitness women and aerobic training that increased VO_2max also did not alter 2-OHE1, 16α-OHE1 or 2:16α-OHE1. These findings suggest that if physical activity does alter estrogen metabolites it may not be mediated by changes in aerobic fitness.

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List of Abbreviations

2-OHE1	2-hydroxyestrone
16α-OHE1	16α-hydroxyestrone
ACS	American Cancer Society
BMD	Bone mineral density
BMI	Body mass index
CCS	Canadian Cancer Society
CYP	Cytochrome
DEXA	Dual-energy x-ray absorptiometry
E1	Estrone
E2	17β-estradiol
E3	Estriol
ELISA	Enzyme-linked immunoabsorbant assay
EMC	Estrogen metabolite concentration (addition of 2-OHE1 and 16α-OHE1
FSH	Follicle-stimulating hormone
HRT	Hormone Replacement Therapy
Kcal	Kilocalories
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LPD	Luteal phase defect
LTPA	Leisure-time physical activity
MET	Metabolic equivalent (measure of energy cost of activity)
OR	Odds ratio

RCT	Randomized controlled trial
RPE	Rating of perceived exertion
SE	Standard error
SHBG	Sex hormone binding globulin
SSF	Sum of skinfolds
V_E	Ventilation
V_E/VO_2	Lowest point in the ratio of ventilation and oxygen consumption prior to a systematic increase without a concurrent rise in the ventilatory equivalent for oxygen
V_E/VCO_2	Lowest point in the ratio of ventilation and carbon dioxide production prior to a systematic increase
VO_2	Oxygen consumption
VO_{2max}	Maximal oxygen consumption as determined by an incremental exercise test
WCRF	World Cancer Research Fund/American Institute for Cancer Research
WHR	Waist-to-hip ratio

CHAPTER 1 - Introduction

Cancer is a significant health problem in Canada with 149,000 new cancer cases and 69,500 cancer deaths expected in 2005. Today, Canadian women have a 38% chance of developing cancer in their lifetime and for Canadian men that chance is 44% (1). Significant research efforts are aimed at determining the causes of cancer and developing prevention strategies. One emerging avenue of research is the role of physical activity in the primary prevention of cancer. Based on the evidence provided by more than 170 observational epidemiological studies, there is a "convincing" association between physical activity and risk of both colon and female breast cancer, a "probable" association with prostate cancer, and "possible" association with endometrial and lung cancer. While links between physical activity and other cancers, such as testicular, ovarian, kidney, pancreas, thyroid and melanoma, have been reported, fewer studies in these cancer types have been conducted and the evidence for an association at this time is "insufficient" (2). The biological mechanisms responsible for the association between physical activity and cancer risk reduction are still under investigation, but have been suggested to include changes in sex steroid and metabolic hormone levels, growth factors, body composition, gut transit time and immune function (2).

One proposed biological mechanism for the observed association between physical activity and cancer risk is estrogen metabolism, specifically 2-hydroxyestrone (2-OHE1) and 16α- hydroxyestrone (16α-OHE1). The majority of literature on estrogen metabolism and cancer has focused on breast cancer, however, there is emerging evidence that estrogen metabolism may play a role in other cancers, such as prostate, endometrial, ovarian and kidney cancer, as well as cancers of the head and neck.

Physical activity has been suggested to alter estrogen metabolism in favour of an increase in 2-OHE1, which in turn has been linked to lower cancer risk. However, to date there are only five prospective observational studies (3-7) (Table 3), four intervention studies using acute bouts of exercise (8-11) and two chronic exercise intervention studies (12, 13) that have investigated the impact of physical activity and estrogen metabolites, 2-OHE1 and 16α-OHE1. The aim of this dissertation is to add to the current literature by examining the effect of physical activity on an identified breast cancer biomarker, estrogen metabolites (2-OHE1, 16α-OHE1 and 2:16α-OHE1) in healthy, normally cyclic, sedentary, premenopausal women.

The early observational studies (4, 6, 7) had small samples sizes (5-7 participants per group), compared participants with and without menstrual dysfunction, and used older techniques for measuring estrogen metabolism that make comparison to more recent literature difficult. The recent observational studies (3, 5) have improved on the methodology of the earlier studies by using larger sample sizes, standardizing menstrual status and analysing estrogen metabolites with a commercially available laboratory procedure. However, all studies have measured physical activity with self-reported measures, such as swim distance, running mileage, participation in a varsity-rowing program, leisure time physical activity or occupational activities. Self-reported physical activity suffers from a number of methodological issues (14), in particular over reporting of frequency, duration and intensity. One of the two chronic exercise intervention studies looked at a year long exercise intervention in postmenopausal women (12), while the other examined the effect of a 20-week lifestyle intervention in premenopausal women, that involved dietary changes in addition to simply encouraging women to be more active (mainly through walking). Physical activity was measured by self-report and accelerometers (13).

Study One aims to examine the association between aerobic fitness level and estrogen metabolites, 2-OHE1 and 16α-OHE1, in premenopausal women. Maximal oxygen consumption (VO_{2max}) was used an objective indicator of chronic physical activity level. Highly aerobically trained women (VO_{2max} ≥48 ml/kg/min) will be compared to sedentary and recreationally active women (VO_{2max} < 40 ml/kg/min) on estrogen metabolites, 2-OHE1, 16α-OHE1 and 2:16α-OHE1. It is hypothesized that the highly aerobically trained women will have higher levels of 2-OHE1, lower levels of 16α-OHE1, and higher 2:16α-OHE1, a pattern consistent with a decrease breast cancer risk.

Study Two aims to examine the effect of a supervised aerobic exercise training program on 2-OHE1, 16α-OHE1 and 2:16α-OHE1 in previously sedentary or recreationally active premenopausal women (VO_{2max} < 40 ml/kg/min), using randomized controlled trial methodology. Participants will be randomized to either a 12-week individualized, progressive, moderate to vigorous intensity aerobic exercise training program, designed to improve aerobic fitness or usual lifestyle group. It is hypothesized that the aerobic exercise training program will cause an increase in 2-OHE1 and 2:16α-OHE1. It is also hypothesized that changes in estrogen metabolites may be associated with change in aerobic fitness and body composition.

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CHAPTER 2 – Review of Literature

1.0 Overview of research question

1.1 Epidemiological evidence for decreased breast cancer risk due to physical activity

In 2005, it is estimated that 21,600 Canadian women will be diagnosed with breast cancer and 5,300 will die from it (1). These numbers point to the need for interventions designed to lower breast cancer incidence. Epidemiologic evidence suggests a 30-40% decrease risk of breast cancer in women who are physically active. Thirty-two of 44 studies that have been conducted to date show a risk reduction in women who were more physically active (2). Two studies found an increased breast cancer risk with increased physical activity (3, 4), and the remaining studies found no association (2). Overall, the evidence of risk reduction associated with physical activity is noted as "convincing" and the risk reduction "considerable" (2). A dose-response relationship has been noted in 15 of 22 studies (5). A review on dose response by Thune and Furberg (6) reported that leisure time physical activity (LTPA) of moderate intensity (4-5 metabolic equivalents or METs) for at least 4 hours per week, or continuous vigorous activity (24.5 MET/h/wk) is needed to reduce breast cancer risk. In addition, recent evidence suggests a reduced risk of recurrence, cancer-specific mortality and all-cause mortality in cancer survivors who are physically active (7, 8).

However, all the current research examining this association is observational. Stronger evidence of a causal effect would be garnered by the use of randomized controlled trial methodology. Intervention studies looking at the impact of lifestyle factors, such a physical activity, on cancer incidence presents numerous feasibility issues (i.e. large sample sizes, lengthy interventions and follow up, and significant financial costs), which has been the main argument for the reliance on observational finding. However, evidence from large scale randomized controlled trials is needed to provide truly convincing evidence of a causal effect and this need may drive the initiation of such an investigation.

1.2 Current physical activity guidelines for reduced cancer risk

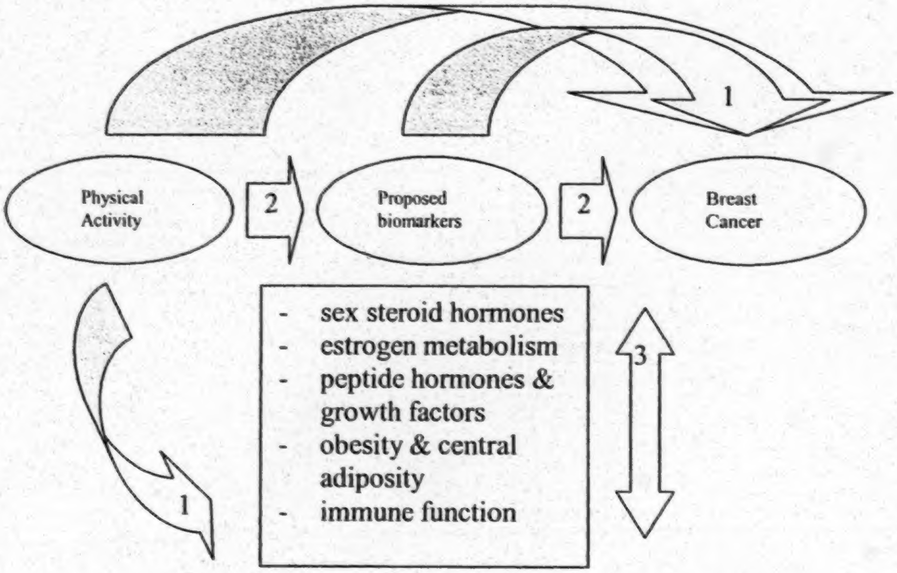
Health Canada (9) and the Center for Disease Control and Prevention (CDC) (10) in the United States have developed physical activity recommendations to promote health benefits for the general population. Those who are currently sedentary can improve health and well being by engaging in regular moderate physical activity. Both agencies recommend moderate intensity activity for 30-60 minutes, on five or more days per week, or vigorous activity for 20-30 minutes, on three or more days per week. These guidelines are also endorsed by the American Cancer Society (ACS) (11) and Canadian Cancer Society (CCS) (12), for reducing cancer risk. In addition, the ACS guidelines suggest 45 minutes or more of moderate to vigorous activity on five or more days per week may further enhance reductions in the risk of breast and colon cancer (11). This appears to be an effective and feasible type of exercise prescription that could be used to investigate the effect of a physical activity intervention on breast cancer biomarkers.

1.3 Understanding the biological mechanisms for an association between physical activity and breast cancer

Despite this convincing epidemiological association, the underlying biologic mechanisms mediating the association between physical activity and breast cancer risk have not been established. Determining these mechanisms not only illustrates biological plausibility but also provides causal data for the association between physical activity and breast cancer risk. Moreover, understanding the mechanisms of reduced risk can help inform the optimal exercise prescription for breast cancer risk reduction. Possible biological mechanisms include changes in endogenous sex steroid hormone production, estrogen metabolism, circulating concentrations of peptide hormones and growth factors, obesity, central adiposity, and immune function (13). Research into these mechanisms aims to provide a basis for physical activity guidelines designed to reduce breast cancer risk for future women and to add more definite evidence on the causal role of physical activity and breast cancer risk. While low physical activity levels do not singularly account for the development of breast cancer, determining how physical activity fits into prevention strategies should prove to be valuable population health information.

However, understanding the association between physical activity and breast cancer is difficult since physical activity is a complex behaviour to measure and the etiology of breast cancer is multifactoral (14-16). A current avenue of research has focused on generating a better understanding of how physical activity influences cancer risk by using biomarkers as surrogate outcomes, rather than the disease itself. Biomarkers are biological factors that are thought to be involved in the causal pathway between exposure and cancer development (16). Examination of biomarkers allows for the determination of events along the causal chain from exposure to disease. Rundle (16) has outlined the case for cancer biomarkers studies involving physical activity. Biomarker studies can be used to 1) test and refine causal hypotheses, 2) elucidate the mechanisms by which activity exerts its protective effects and 3) provide a useful surrogate outcome in intervention trials which test physical activity prevention programs.

For breast cancer, observational associations between: 1) physical activity and breast cancer, 2) proposed biomarkers and breast cancer, and 3) physical activity and proposed biomarkers, have been demonstrated, however, causal links are less clear (Figure 1). In addition, the causal ordering of proposed biomarkers and how biomarkers may interaction with each other to enhance or negate the action of another is also not well understood at this time.



1= Demonstrated observational associations

2 = Some causal effect demonstrated

3 = Causal ordering and "cross talk" currently under investigation

Figure 1 – Association between physical activity, estrogen metabolism and breast cancer

Estrogen metabolites 2-hyroxyestrone (2-OHE1) and 16α-hydroxyestrone (16α-OHE1) are biomarkers of interest for breast cancer. An observational association between physical activity and breast cancer risk has been well documented (2). Observational associations between proposed biomarkers associated with physical activity and breast cancer risk, in this case estrogen metabolism has also been shown (17-24). Overall, epidemiological studies, including both case-control and prospective cohort studies, are generally supportive of the association between estrogen metabolism and breast cancer risk, but limited in number, and quality, while biological plausibility has been established via *in vitro* and animal models. However, further research into the causal associations between physical activity and estrogen metabolites is needed. To date the research on alterations in estrogen metabolism due to physical activity has been limited in scope and suffered from a number of methodological problems. Improved methodology such as the use of objective measures of physical activity and randomized controlled trials of physical activity are needed.

The aim of the two studies in this dissertation is to add to the understanding of the causal effects of physical activity on a proposed breast cancer biomarker, estrogen metabolites (2-OHE1 and 16α-OHE1) in healthy, normally cyclic, premenopausal women. The first study uses an objective quantification of aerobic fitness, as a surrogate measure of usual physical activity, in a cross-sectional study to compare estrogen metabolites in highly aerobically fit women to those of average aerobically fit women, while the second study examines the effect of a supervised aerobic exercise training intervention, designed to improve aerobic fitness, on these estrogen metabolites.

1.4 Implications for pre- versus postmenopausal breast cancer risk

Breast cancer incidence increases with age. The risk of developing breast cancer for women 30-39 is 4 out of 1000 before age 40, 13 out of 1000 for women age 40-49 before age 50, 25 out of 1000 for women age 50-59 before age 60, 31 out of 1000 in women age 60-69 before age 70, 33 out of 1000 in women age 70-79 before age 80 and 26 out of 1000 in women age 80-89 before age 90 (1). Over 80% of breast cancer diagnoses occur in women over the age of 50, which is commonly assumed to be classified as postmenopausal breast cancer (25). However, both the presentation and proposed risk factors of pre- and postmenopausal breast cancer appear to vary. Commonly cited breast cancer risk factors may now be associated more with pre- (early age of menarche, and later age of first full-term pregnancy) or postmenopausal (later age at menopausal, number of full-term pregnancies) breast cancer risk (26, 27). While physical activity may act differentially in pre- and postmenopausal breast cancer risk, physical activity in the premenopausal years has been reported to decrease both pre- (28) and postmenopausal breast (29, 30) and physical activity later in life (i.e. after menopause) has also been shown to lower subsequent breast cancer risk (30).

The majority of studies (22 of 27) in postmenopausal women show an association between greater physical activity and reduced risk of breast cancer (27). In premenopausal women the association is not as clear, with no association between physical activity level and breast cancer risk in 12 of 26 studies. Of the remaining studies in premenopausal women, half found a statistically significant decrease in risk (7 of 14), while the remainder found a non-significant decrease in risk (6 of 7) or a non-significant increase in risk (1 of 7) (27).

A review by Friedenreich (27) outlines the contribution of physical activity on risk factors for breast cancer by menopausal status at the time of diagnosis, divided into pre- and postmenopausal breast cancer. Weight maintenance, weight loss and reduction in adiposity are proposed for a reduced risk of postmenopausal breast cancer due to physical activity. While a higher body mass is thought to have a protective effect for premenopausal breast cancer, physical activity can still influence other factors associated with high body mass, such as insulin resistance, sex hormone binding globulin levels, and markers of inflammation that have also been linked to breast cancer risk (27). As a result, physical activity may act across the lifespan to slightly reduce the risk of premenopausal breast cancer, and more substantially reduce risk of postmenopausal breast cancer.

A similar argument can be used for altering the pattern of estrogen metabolism, to favour 2-OHE1 production, as is suggested to occur with physical activity. Alteration in the premenopausal years would decrease total lifetime estrogen exposure and would, in turn, contribute to a potential reduction the lifetime risk of breast cancer. In addition, along with physical activity alone, the association between body composition and estrogen metabolites provides an argument for physical activity in the both the pre- and postmenopausal years to limit further exposure to estrogens and shift estrogen metabolism towards a more favourable 2:16α-OHE1 to reduce the risk of breast cancer.

2.0 Methodological Issues

2.1 Trial methodology

While a variety of research methodologies exist, the research question guides the decision of which methodology is the most appropriate. In observational studies, participants are observed but no attempt is made to affect the outcome and this approach is often used when less is known about the association of interest or as the basis for subsequent experimental designs, or when logistical issues are present. One type of observational study, which was used in Study One of the dissertation, is a cross-sectional design in which there is a simultaneous assessment of the exposure and outcome of interest. This allows for determination of the pattern of the outcome in an identified group. This type of study is efficient, in that it samples data at a single point in time, but is not able to establish causality by demonstrating cause and effect (31, 32).

Randomized controlled trial (RCT) methodology, which was used for Study Two of this dissertation, is a prospective experimental research design that affords the strongest evidence for causality in epidemiological research and approximates the controlled experiment of basic science (31). Participants are randomly assigned to an intervention group or non-intervention control group (i.e. usual lifestyle, placebo, standard care) and compared on an outcome of interest following the intervention. Random allocation is the hallmark of this research design and needs to be executed rigorously (33). This strengthens internal validity of the trial (i.e. it eliminates alternative explanations for the findings) and avoids selection and confounding biases (31, 32). In addition, randomization provides comparable groups at baseline, which are indirectly matched on both identified and unidentified factors associated with the outcome of interest (34). However, external validity (i.e. the generalizability of the findings to the general population) may be hampered by the fact that participants are volunteers and by the rigorous screening processes often used for such trials. However, the ability to undertake studies with greater generalizability may be limited for some research questions, such as when intentional exposure could be harmful and unethical or when outcomes have long latency periods and studies are prohibitively expensive (31). In addition, proper reporting of RCTs, as outlined by the CONSORT group (35), is essential to avoid biases in determining the effectiveness of the intervention and to allow readers to judge the quality of the trial.

2.2 Measuring physical activity

The impact of physical activity on the risk of various diseases is an important area of study, however, achieving a valid and reliable method for measuring physical activity is difficult. This is primarily due to the complex components of physical activity, namely intensity, duration, frequency, mode of activity, surrounding environment and social conditions that need to be determined to provide an accurate assessment of physical activity behaviour (36, 37).

By definition, physical activity is "any bodily movement produced by skeletal muscle that results in energy expenditure". Exercise "is physical activity that is planned, structured, repetitive" and with the intent to improve or maintain physical fitness. Physical fitness is "the ability to carry out daily tasks with vigour and alertness, without undue fatigue and with ample energy to enjoy leisure time pursuits and to meet unforeseen emergencies". In addition, health-related physical fitness is "the state of physical and physiological characteristics that define the risk levels for the premature development of diseases or morbid conditions presenting a relationship with a sedentary mode of life" (38). Health-related fitness can be broken down into: (1) a morphological component (body mass index, body composition, subcutaneous fat distribution, abdominal visceral fat, bone density and flexibility); (2) a muscular component (power or explosive strength, isometric strength, muscular endurance); (3) a motor component (agility, balance, co-ordination, speed of movement); (4) a cardiorespiratory component (endurance or submaximal exercise capacity, maximal aerobic power, heart function, lung function, blood pressure); and (5) a metabolic component (glucose tolerance, insulin sensitivity, lipid and lipoprotein metabolism, substrate oxidation characteristics) (38). These definitions are important when discussing how physical activity impacts health risk. Is physical activity in and of itself enough to produce positive health outcomes for a specific condition or are improvements in various aspects of health-related fitness needed to achieve these benefits?

Vanhees et al. (39) have outlined three main types of physical activity assessment methods as criterion methods, objective methods or subjective methods. Each has benefits and drawbacks based on the research question and the population under study. Criterion methods, such as doubly-labelled water, indirect calorimetry and direct observation are the most reliable and valid measurements, and the measures against which other physical activity assessments methods should be validated. However, these are often expensive, labour intensive and unsuitable for large field studies. Objective physical activity assessment methods, such as activity monitors (i.e. pedometers and accelerometers) and heart rate monitoring still allow for quantification of various aspects of physical activity and are more feasible in the field setting. However, these have inherent drawbacks related to their ability to measure movement in all planes of motion and debate over how the movement recorded is translated into measures of physical activity or energy expenditure (39). Questionnaires and activity diaries are considered subjective methods, which are convenient for sampling large numbers of people, however, with self-report measures participants tend to overestimate moderate-to-vigorous physical activity and are poor at reporting activities that were unorganized, less vigorous or used as transportation (40).

Ideally, when determining the relationship between physical activity and a specific outcome of interest (i.e. biomarker of disease risk), the most accurate measure of physical activity should be used. Doubly-labelled water allows for the determination of energy expenditure by measuring the elimination rates a standardized ingested amount of two stable isotopes (^2H and ^{18}O) as water ($^2H_2{}^{18}O$) and is considered the gold standard for measuring energy expenditure in free-living individuals (39 951). However, this technique is expensive which limits its use for large-scale studies and only measures overall energy expenditure, so cannot distinguish between energy expenditure due to physical activity, dietary thermogenesis or resting metabolic rate (41). Indirect calorimetry during an incremental exercise test can be used to determine cardiorespiratory fitness or maximal oxygen consumption (VO_{2max}), and is another criterion measure of habitual physical activity (39). While a genetic contribution is thought to account for 35-50% of an individual's VO_{2max}, regular participation in aerobic activity also makes a large contribution to VO_{2max} and accounts for the variability seen in the population (42, 43). While this is an indirect measure of habitual physical activity levels, it avoids the measurement issues of objective measures, such as accelerometers, and subjectivity seen with self-reported measures.

Measurement of physical activity in women has traditionally had additional associated challenges. Women tend to spend significant time in occupational, household and family-care activities, and less time on recreational or exercise activities, with the result that subjective measures of physical activity do not accurately capture the activity of women (44). In addition, Masse et al. (45) suggest that women are more likely to engage in unstructured, intermittent and simultaneous physical activity, often of moderate intensity, all aspects of physical activity that are traditionally difficult to assess. While identification of these issues will serve to improve future measurement of physical activity in women, they may have hampered previous association studies looking at physical activity levels and disease risk in women.

2.3 Measuring sex steroids

A number of methodological considerations exist when sampling hormones, especially in reference to physical activity. A review by Tremblay and Chu (46) outlines a number of these factors. Measurement issues are particularly crucial in women due to the cyclic nature of sex steroid hormones in premenopausal women. Age and maturation, ethnicity, body composition, general health (medical conditions, surgery and medication use), mental health, recent physical activity, and circadian rhythm have all been shown to alter endocrine profile (46). As a result, determining eligibility criteria and standardization of sample collection is key. Frequent sampling of all relevant factors would give investigators very valuable information, however, this could potentially be overly burdensome to participants, and costly to carry out. In an attempt to determine the validity of less frequent sampling, investigators have attempted to determine the optimal sampling procedures. Ahmad et al. (47) obtained alternate day venous blood samples over one menstrual cycle in 24 healthy premenopausal women. The authors suggest that a single blood sample between day 9-11 for estradiol, day 12-15 for androgens (i.e. testosterone, and androstenedione), and day 17-21 for progesterone is likely to give a reasonable characterization of inter-individual differences (47).

The occurrence of ovulation is an important biomarker of reproductive function, and is a valuable tool for determining the presence of subtle menstrual disturbances (48). While the instance of anovulation in the general population of eumenorrheic women appears to be low (3.7%) (49), reports of a higher incidence of anovulation associated with physical activity have been reported (50). While the most accurate method for detecting ovulation is ultrasonic observation of follicular growth and rupture, the cost and required skill level make this method prohibitive. As a result, measurement of luteinizing hormone (LH) concentration in midcycle serum or urine, or measurement of daily luteal phase urinary steroid metabolites are commonly used (48). Several valid algorithms using these measures have been developed to determine the presence and day of ovulation (48). If daily urine samples are not feasible, Williams has suggested determination of ovulation by achieving a critical threshold value on two of three samples (day 19, 21, and 23) (personal communication). However, this strategy is problematic if cycle length is lengthened beyond average cycle length (i.e. 28 days).

2.4 Measuring 2-OHE1 and 16α-OHE1

Early attempts at measuring estrogen metabolites required administration of stereospecifically labelled estradiol (51). However, the development of a sensitive and specific immunoassay by Klug, Bradlow and Sepkovic (52) provided a less invasive way to measure these estrogen metabolites. The newest version of the ELISA procedure has shown good agreement with gas chromatography mass spectroscopy (53, 54). Reported intra- and inter-assay variation for 2- and 16α-OHE1 range from 4.0-12.0% (55, 56) and stability of metabolites that have been frozen for more than a year at -80°C has also been noted (56).

Within-person variability for urinary 2:16α-OHE1 appears to be low, and correlations are noted between a single urine sample and the average ratio of weekly samples across eight weeks (57). Westerlind (56) noted that while absolute values of each metabolite varied across the menstrual cycle, no significant difference in the ratio was observed. However, Xu (58) noted an increase in 2:16α -OHE1 from the early and midfollicular phases, to the periovulatory and midluteal phases. This is in agreement with Chen (59) who found that 2:16α-OHE1 was more than 50% higher in the luteal phase, along with higher overall levels of both 2-OHE1 and 16α-OHE1 in the luteal compared to the follicular phase. Overall, controlling for phase of menstrual cycle appears to be an important consideration when examining estrogen metabolites.

Measurement of 2-OHE1 in serum using the commercially available immunoassay kit has been published (60). However, a recent investigation comparing 2-OHE1 levels found in urine and serum using the commercially available immunoassay kit, found lower overall levels compared to those found in urine, high within-run coefficients of variation (20%), higher among-run coefficients of variation (30%) and some interference by estriol levels (61). This suggests that further research is needed to determine the validity and reliability of measurement of estrogen metabolites in serum.

3.0 Role of sex steroid hormones in breast cancer risk

Cumulative lifetime exposure to sex steroids, particularly estrogens, is thought to play an important role in breast cancer risk (62-64). The impact of other sex steroid hormones, particularly progesterone and testosterone, on breast cancer risk have been reviewed by Travis and Key (65) and Bernstein (66).

3.1 Estrogens

A vast body of literature has implicated estrogens in the etiology of breast cancer. The majority of identified breast cancer risk factors are attributed to cumulative lifetime estrogen exposure. Aspects of reproductive history, including early menarche, later menopause, and older age at first full term pregnancy, as well as postmenopausal obesity and the use of exogenous hormones have been implicated (65-67).

Hormones are blood-borne chemical messengers that are secreted by endocrine glands in response to specific signals. One class of hormones are steroid hormones. In order to exert their effect, steroids must bind to target-cell receptors (68). Estrogens, which are steroid hormones, play an important role in the normal development of secondary sex characteristics and reproductive function in females (65). In relation to breast tissue, estrogens simulate growth and differentiation of ductal epithelium and growth of connective tissue. However, by stimulating ductal growth and cell proliferation rate in the breast, estrogens also increases the likelihood of the occurrence of a random genetic error (66).

The estrogens, estrone (E1), 17β-estradiol (E2), and estriol (E3), are steroid hormones derived from cholesterol (69). The main source of estradiol in premenopausal women is the interstitial cells of the ovaries, while estrone and estriol are primarily formed in the liver from estradiol (69). Cholesterol binds to lipoprotein receptors, and is then taken up by steroidogenic cells. A subsequent reduction of the number of carbon atoms from 27 to 18 results in the formation of different steroids. The transfer of cholesterol from the cytosol to the inner membrane of the mitochondrion is the rate-limiting step in steroid production. In the mitochondria, cytochrome P450 enzymes catalyze the cleavage of the side chains of cholesterol (Figure 2). Polymorphisms in genes which code enzymes along the biosythesis pathway, such as CYP17 and CYP19, have been linked to altered levels of estrogens (62). The final step in the formation of estrogens is aromatisation, catalyzed by the P450 aromatase enzyme complex in the smooth endoplasmic reticulum (69). The result is the formation of estrone and estradiol from their respective precursors, androstendione and testosterone (69). Most of the estradiol in the body is reversibly bound to either sex hormone binding globulin (SHBG), or with lesser affinity to albumin, leaving only 2-3% "free" estradiol.

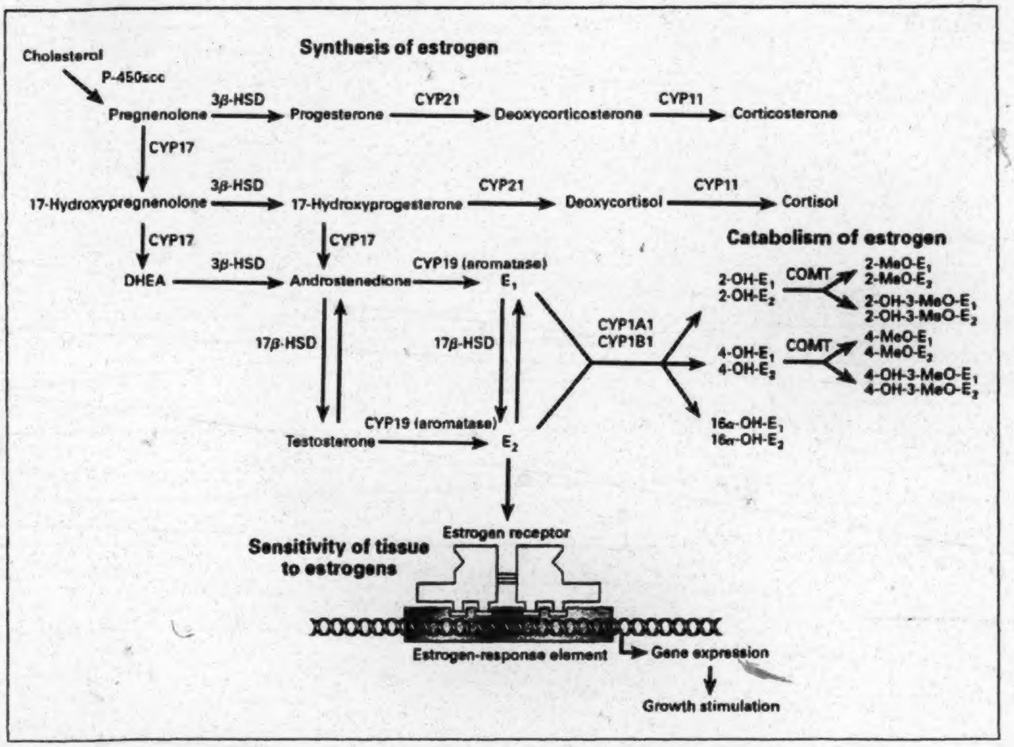


Figure 2 – Pathways of estrogen synthesis and metabolism (62)
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Metabolism of estrogens by sulfation, glucuronidation, hydroxylation and/or O-methylation, produces water-soluble metabolites that are excreted into bile or urine (70). Hydroxylation results in 2-hydroxyestrogens (2-OHE), 4-hydroxyestrogens (4-OHE), and 16α-hydroxyestrogens (16α-OHE) (69).

In discussions of breast cancer risk, the type of estrogen, i.e. estrone, estradiol and estriol, is often unspecified. However, when specified, estradiol is the predominant estrogen that has been linked to breast cancer risk (62). Levels of estrone have also been linked to breast cancer risk, while the estrogenic effect of estriol appears to be low and a proposed protective effect against breast cancer has not been confirmed (71, 72).

The following are factors that may influence lifetime exposure to estrogens:

3.1.1 Menarche

Young women who experience menarche before age 12 have a risk of breast cancer that is twice that of women whose age of menarche is 13 or older (73). In fact, a reduction of 5-15% has been proposed for each later year of age at menarche (74). Physical activity in adolescence may delay the onset of menarche, thus reducing the number of ovulatory cycles and overall lifetime exposure to estrogens (75). Others have argued that rather than physical activity altering age at menarche, girls who self-select into sports may be those who mature later, because later maturation may offer performance advantages, whereas girls who mature earlier may be socialized away from athletics (76). However, the majority of research on the impact of physical activity on age of menarche is hampered by its retrospective and observational nature (77). Later age at menarche has also been associated with a longer interval until onset of regular menstrual cycles, which would also decrease overall lifetime exposure to estrogen (78).

3.1.2 Menopause

A further reduction in cumulative estrogen exposure is associated with early menopause. A higher number of lifetime menstrual cycles is associated with increased postmenopausal breast cancer risk (79). Women who experience natural menopause before age 45 have half the breast cancer risk of women whose menopause occurs after age 55 (73, 80). Surgically induced menopause, (i.e. oophorectomy) also has a protective effect, especially if it occurs before age 45 (73). However, physical activity has not been shown to affect age at menopause (64).

3.1.3 Parity

Parity and age of first full term pregnancy appear to impact ovarian hormone exposure. A woman with a full term pregnancy before age 19 has half the risk of developing breast cancer, as compared to a nulliparous woman (25). The risk is equal to that of nulliparous women, if the first full term pregnancy is between ages 30-34 years. It is thought that full term pregnancy induces differentiation of the breast and this may alter the sensitivity of the breast to hormonal risk factors (81, 82). Clavel-Chapelon and the E3N-EPIC Group (82) found that overall parity also influenced risk with each full term pregnancy decreasing postmenopausal breast cancer risk by 8% up to a parity of four or more. Overall parity may impact breast cancer risk by decreasing overall ovarian hormone exposure related to the hormonal changes associated with interruption of normal menses during gestation and subsequent breast feeding (82). Parity may also result in long term changes in estrogen levels. In postmenopausal women with four or more children, lower estradiol levels and higher SHBG levels were found compared to women with only one child (83).

3.1.4 Body mass and body composition

After menopause, the primary source of estradiol is production through conversion of androstenedione and estrone by adipose tissue (62). Higher body mass index (BMI), higher body mass, adult body mass gain and waist to hip ratio (WHR) have been associated with an increased postmenopausal breast cancer risk (84-87). Obesity has been shown to result in increased plasma concentrations of testosterone, decreased concentrations of SHBG, and subsequently increased levels of free estradiol (84). Increased abdominal fat distribution or central fat distribution has also been reported to increase the risk of postmenopausal breast cancer. Risk in those with more abdominal adiposity was increased from 1.4 to 5.2 times that of women with less central body fat (88). Waist circumference, which is a measure of central fat distribution, has been shown to be a stronger predictor of breast cancer risk than WHR, both of which are better predictors than BMI (84, 89). Adult body mass gain has also been implicated, as this is usually indicative of an increase in body fat, and is therefore a better measure of adiposity than body mass alone (84, 90). Adding to the association of body mass and breast cancer risk is the observation that body mass loss, especially later in life, decreases postmenopausal breast cancer risk, presumably by lowering overall estrogen exposure by reducing the conversion of androstenedione and estrone by adipose tissue (91). Physical activity has been successful in preventing adult body mass gain, encouraging body mass loss to achieve a healthy body mass, and reducing central adiposity (92, 93).

A recent breast cancer prevention intervention trial in overweight or obese postmenopausal women showed that, in those randomized to a 12-month moderate-to-vigorous intensity aerobic activity program, there was a reduction in body mass (1.4 kg) and body fat (-1%) (94), as well as declines in estrone (3.8%), estradiol (7.7%), and free estradiol (8.2%) compared to either no change or increases in hormone concentrations in controls at 3 months (the direction of effects remained the same at 12 months but were no longer statistically significant) (95). In fact, the greater the decrease in percent body fat, the greater the decrease in estrogen levels (95).

In premenopausal breast cancer, higher body mass has consistently been shown to be protective for breast cancer incidence (84), but if breast cancer develops, obesity has been associated with increased risk of recurrence and mortality (96). A pooled analysis of prospective cohort studies by van den Brandt et al. (97) reported an inverse association for premenopausal women weighing more than 80 kg (OR = 0.58;95% CI 0.40–0.83). A similar association was reported for BMI, in that premenopausal women with a BMI greater than 31 kg/m ^{2} had a lower risk than those with a BMI of less than 21 kg/m ^{2} (OR = 0.54; 95% CI 0.34–0.95). A higher frequency of anovulation, seen with higher body mass and BMI, is suggested to contribute to this risk reduction, as this would lower estrogen exposure in premenopausal women (15). Overall, obesity in premenopausal women has been associated with lower concentrations of estrogen, progesterone and SHBG, as well as higher concentrations of testosterone (84).

3.1.5 Ethnicity

Differences in breast cancer incidence have been documented across ethnic groups (98). Comparison of hormone level by ethnicity was undertaken as part of the large Nurses Health Study cohort (99). In premenopausal women, African Americans had higher levels of estradiol (18%) and free estradiol (17%) levels compared to Caucasian women. This is consistent with the increased observed risk of premenopausal breast cancer in African American women. However, Asian American women also had higher free estradiol (22%) compared to Caucasian women, which is inconsistent with the proposed association between estradiol and breast cancer risk, since Asian American women have a lower risk of both pre- and postmenopausal breast cancer than Caucasian women (99).

3.1.5 Exogenous hormones

Exogenous hormones have also been implicated, notably oral contraceptives and hormone replacement therapy (HRT). The impact on oral contraceptive use on breast cancer risk remains controversial (100). A review by Persson (63), showed a 25% increase in risk of breast cancer, with 5 years or longer use of oral contraceptives, in women under age 35. An increased risk was also seen with use before age 20 or first full term pregnancy. Notably, five years after cessation of use, no increased risk was seen (63). However, a recent case control study of 4575 women with breast cancer, and 4682 controls, in the United States, showed no increase risk related to use or duration of use of oral contraceptives (101).

HRT and breast cancer risk is also quite controversial (102). The Women's Health Initiative reported an increased risk of breast cancer following the use of combination HRT (including estrogen and progestins) but a nonsignificant decrease in conjugated equine estrogen alone (103, 104). A review by Persson et al. (63) also found an increased relative risk ranging from 1.5 to 3, after 6-10 years of HRT use. The increase in risk seems to be dependent on duration of use and the risk has been reported to disappear five years after discontinuing use (63).

3.1.6 Chemoprevention agents

The identified risk factors for estrogen-dependent cancers, such as familial susceptibility, menstrual cycle characteristics, reproductive behaviours and socio-economic status, are not readily alterable (105). While current strategies for breast cancer management rely on early detection, moving towards primary prevention could decrease the disease burden significantly. This has led to attempts to block the activity of estrogens with pharmacologic agents such as tamoxifen (106), and raloxifene (107). The most recent advancement is the aromatase inhibitor, Letrozole, which prevents the production of estrogens by peripheral conversion of androgens (e.g. conversion of testosterone and androstenedione to estradiol and estrone, respectively), the major source of estrogens in postmenopausal women (108). However, the use of these chemoprevention agents is not without significant side effects, such as hot flashes, vaginal bleeding, musculoskeletal pain, venous thromboembolism, stroke, osteoporosis and related fractures (107, 109).

3.2 Progesterone

Progesterone has been proposed to enhance the effects of estrogens on breast cancer development (i.e. "estrogen-plus-progesterone" hypothesis) by binding to their respective receptors and thus promoting proliferation of breast epithelium (110). This is based on observations that breast cell proliferation is greatest during the luteal phase, where progesterone dominates, and that mitotic activity in the terminal ductal lobular unit of the breast is higher in the breast biopsy samples of women who are taking combined estrogen and progesterone hormone replacement therapy preparations, than in women who are taking estrogen alone (65). Progesterone has been reported to be a significant determinant in the initiation of mammary tumours secondary to carcinogens in animal studies (111). Overall, the literature on the effects of endogenous progesterone in premenopausal women and overall breast cancer risk is inconsistent. However, a recent study reported that higher premenopausal progesterone levels were associated with a reduced breast cancer risk (112). In terms of postmenopausal breast cancer, an increase in risk has been associated with the addition of progesterone to estrogen replacement regimes (66, 113). However, the precursors of many synthetic progestins are androgenic molecules complicating the findings of this observation (112).

3.3 Testosterone

Exposure to androgens, specifically testosterone and androstenedione, can directly increase the growth and proliferation of breast cancer cells and has also been suggested to indirectly to increased estrogenic exposure by conversion to estrogens in normal breast tissues and breast tumour specimens (112, 114). In addition, serum testosterone levels have been positively associated with breast cancer risk in most studies that have investigated this association (66). A recent study has confirmed these findings, with higher premenopausal levels of testosterone associated with an increased breast cancer risk (112). For postmenopausal women, a re-analysis of nine prospective studies reported that women in the top quintile of testosterone levels had double the risk of developing breast cancer, as that of a woman in the lowest quintile (115).

3.4 Levels of Evidence for the association between sex steroid hormones and breast cancer

Currently, researchers and clinicians have access to an ever increasing body of literature. Techniques to combine information on a particular topic, such as systematic reviews (i.e. Cochrane reviews) and meta-analysis, and procedures to rate the current evidence have been developed to assist with synthesizing the available literature. Under this model, it is "important, whenever possible, to base firm recommendations on results of rigorously controlled investigations and to be much more circumspect, when recommendations rest only on the results of uncontrolled clinical observations" p.2S (116).

One common rating system is Sackett's Level of Evidence. The most recent published version appears in the text, *Evidence-based Medicine(EBM): How to practice and teach EBM* (117). Evidence from clinical studies can be rated from level of evidence 1-5, and receives a resulting grade of recommendation from A-D. The highest rating is reserved for systematic reviews of randomized controlled trials (RCTs), while the lowest is afforded to expert opinion without explicit critical appraisal. This system can be used to examine several types of questions, such a therapy outcomes, etiology, prognosis, diagnosis or economic analysis of various health related questions.

While Sackett's Level of Evidence is a good fit for the medical model, for epidemiological evidence an alternate model to assess the strength of current evidence has been developed by the World Cancer Research Fund/American Institute for Cancer Research (WCRF) (118). In this model, the summary of research can lead to "convincing", "probable", "possible", or "insufficient" rating of the association under investigation. "Convincing" evidence results from conclusive epidemiological evidence, namely: a consistent association from at least 20 studies that include prospective designs, varied populations, controlling for possible confounding factors, with exposure data prior to cancer occurrence, evidence of a dose response, and biologic plausibility. It is important to note, that this rating system does not require RCT evidence to receive the highest rating. This point is an essential divergence in the rating of evidence using the medical and epidemiological model. Labelling evidence as "convincing" based on observational research alone could be viewed as less stringent than what is required to establish causality.

3.4.1 Estrogens

Previously, Sackett's Level of Evidence has been applied to the question, "Does physical activity lower the risk of primary estrogen related cancers in women" (119). For postmenopausal women, a pooled analysis of nine prospective studies provides the best evidence of a potential association between breast cancer and estrogen (115). A total of 663 cases were identified, and were found to be significantly related to increased concentrations of estradiol, free estradiol, and estrone in blood samples. In fact, high serum estradiol concentration resulted in approximately a two-fold increase in risk compared to low levels. These findings were similar using urinary measures (71). Therefore, higher concentrations of estrogens, particularly estradiol, are suggestive of an increased breast cancer risk in postmenopausal women (Level of Evidence 2a; Grade of Recommendation B). The link between estrogens and postmenopausal breast cancer risk is "probable", meaning that the evidence is strong enough to conclude that a causal relation is likely, but the number of reported studies is not high enough to garner "convincing" status.

The published evidence for such a link in premenopausal women is sparse, with only six prospective studies (112, 120-124). Studies in premenopausal women are often more difficult to execute since many large studies rely on a single sample, which may not be representative of the average circulating level of a sex steroid hormone, due to cyclic fluctuations. These studies contain over 180 breast cancer cases and were matched in varying ratios to cohort controls. Three studies found no difference in estradiol level between cases and controls (112, 122, 123), while two found an insignificant higher mean estradiol level in cases (124), and one study found significantly elevated levels of bioavailable estradiol, and an insignificant increase in total estradiol in cases compared to controls (120). Overall, few conclusions can be drawn but estradiol levels do not appear to significantly differ between in premenopausal cases compared to controls (Level of Evidence 4; Grade C). In premenopausal women, the evidence linking estradiol levels to breast cancer risk is "insufficient", due to the few studies, difficulties in adjusting for the day of menstrual cycle, and conflicting findings.

3.4.2 Progesterone

The mention of a link between progesterone and breast cancer risk is commonly attributed to a review by Pike et al. (110). The review proposes that both estrogen and progesterone play important roles in breast cancer etiology. However, a review by Bernstein (66) notes that the literature regarding the effects of endogenous progesterone on breast cancer risk is confusing and inconsistent.

In postmenopausal women, the research into hormone replacement therapy (HRT) has provided the best information on the effect of progesterone on breast cancer risk. The published observational studies were critically evaluated by Santen (125). Based on stringent criteria, five studies were identified, in which four showed an increase risk of breast cancer in women who used combination HRT, compared to estrogen alone. These studies were a mix of cohort and case-control studies. The recent findings of the

Women's Health Initiative (103), provide information from the first prospective randomized controlled trial (RCT) to address this question. This study found a 5.5% yearly increase in risk of developing breast cancer with combined HRT. While critics have pointed to methodological issues and discrepancies in the analysis, this study provides the best clinical evidence of the effect of progesterone on postmenopausal breast cancer risk. Overall, there is suggestion that progesterone may increase breast cancer risk in postmenopausal women (Level of evidence 1b-3b, Grades A and B). Progesterone and postmenopausal breast cancer shows a "possible" causal association, suggesting that a causal relationship exists but recognizing that the studies are based on exogenous progesterone.

Few studies have examined progesterone levels and breast cancer risk in premenopausal women, and the majority of studies have not reported ovulatory status. It is often impractical for epidemiology studies to catch progesterone peak or average luteal phase level, since daily samples during the luteal phase are required. Of six prospective case control studies nested in larger cohort studies, three found no difference between cases and controls (123, 124, 126), two showed lower progesterone levels in cases compared to controls (112, 127), and one suggested higher median follicular and luteal phase levels in cases but this was not statistically significant (122). However, the most recent studies all report a trend towards risk reduction with higher progesterone levels, when comparing those in the lowest tertile or quartile to those in the highest (112, 126, 127). Overall, the evidence linking progesterone levels to premenopausal breast cancer, is contradictory but suggests that higher progesterone levels may lower breast cancer risk (Level of Evidence 2b; Grade B). In premenopausal women, the evidence for an association between progesterone and premenopausal breast cancer is "possible", but further research is needed for confirmation.

3.4.3 Testosterone

Testosterone may play a role in the etiology of breast cancer (66). The proposed mechanism is that both testosterone and another androgen, androstenedione, can be aromatized into estrogens in granulosa cells of the ovary and in adipose tissue.

For postmenopausal women, the recent pooled analysis of nine prospective studies provides the best evidence of a potential association between breast cancer and testosterone (115). Women in the highest versus the lowest quintile for testosterone level had more than double the risk of breast cancer. Higher testosterone levels are associated with an increase in breast cancer risk in postmenopausal women (Level of Evidence 2b, Grade B). The link between testosterone and breast cancer risk, is "probable" for postmenopausal women, due to the strength of the evidence

Research in premenopausal women in limited to three prospective studies. Two studies found no difference between cases and controls (122, 124), while the most recent study found an increase in risk of breast cancer associated with elevated serum testosterone levels (OR for highest to lower quartile 1.73, 95% CI 1.16 to 2.57; P_{trend} = .10) (112). There is a suggestion that higher testosterone levels are linked to breast cancer risk in premenopausal women (Level of Evidence 2b, Grade B), however, more evidence is needed since there are few studies and the majority show no association between testosterone and breast cancer risk. Overall, the evidence is "insufficient" in premenopausal women, due to the few studies and methodology concerns.

4.0 Role of estrogen metabolism in cancer risk

There is evidence to suggest that estrogens themselves do not account exclusively for the actions the hormone exerts on the body. Estrogen metabolism has been the focus of an increasing amount of research which suggested that these metabolites are not simply inactive excretion products (72). Estrogen metabolites, 2- and 16α-hydroxyestrone (OHE-1) have been linked to cancer, such as breast, endometrial, cervical and prostate, and other health conditions, such as rheumatologic conditions, osteoporosis and cardiovascular disease (72, 128). Zhu and Conney (70) propose that estrogen metabolites may have important biological effects that have yet to be recognized. In addition, Lippert et al. (72) have stated that it is "not meaningful to ascribe an estradiol effect solely to estradiol without measuring its metabolites." However, there are methodological issues surrounding the measurement of estrogen metabolites. Early attempts at measuring estrogen metabolites required administration of stereospecifically labelled estradiol (51), prior to the subsequent development of a specific immunoassay to provide a less invasive way to measure these estrogen metabolites (52, 54). However, it is difficult to compare results from studies that have used these different methodologies to measure estrogen metabolites.

4.1 Overview of estrogen metabolism

Estrogen metabolism takes place almost exclusively by oxidation. The first step in metabolism is the transformation of estradiol to estrone, by oxidation at the C17 position (Figure 2). This process is reversible but the reaction normally favours the formation of estrone since the reverse reduction of estrone to estradiol occurs much more slowly. Estrone is further metabolized in two different ways. Hydroxylation at the Aring produces 2-hydroxyestrone (2-OHE1) and 4- hydroxyestrone (4-OHE1), while hydroxylation at the D-ring produces 16α- hydroxyestrone (16α-OHE1) and estriol. The two pathways are catalyzed by different enzyme systems and cannot be reduced back to estrone (72, 129). Other metabolites have been identified, but by comparison occur in only small quantities (70). The proposed effects exerted by estrogen metabolites are thought to be mediated by both classical estrogen receptors but also specific intracellular estrogen receptors (70).

The majority of estrogen metabolism occurs in the liver by members of the cytochrome P450 family of enzymes. Regulation of cytochrome P450 enzymes in the liver by genetic and environmental factors has been reported, and considerable variation in levels of cytochrome P450 enzymes have been found in human liver samples (70). Isoforms of these cyctochromes are also selectively expressed in extrahepatic tissues (70). It is proposed that formation of estrogen metabolites in target tissues or cells may impact specific biochemical events taking place in those target cells (70).

16α-OHE1 has been shown to be estrogenic, while 2-OHE1 is non- or anti-estrogenic (130). 4-OHE1 is thought to have estrogenic effects, but occurs in the body in very small amounts, and there is less research into its role in cancer risk (72). The estrogenic effects of 16α-OHE1 were again illustrated in a recent study where incubation of ovarian cell lines with 16α-OHE1 showed that 16α-OHE1 caused greater proliferative and anti-apoptotic effects than estradiol itself (131).

The majority of research into the link between breast cancer and estrogen metabolism has focused on 2-OHE1 and 16α-OHE1 (72). Overall, 2- and 16α-hydroxylation are competitive pathways, so an increase in one metabolite occurs at the expense of the other (21, 132). Higher 2-OHE1 levels are suggested to have a protective effect, along with a higher 2:16α-OHE1, while higher 16α-OHE1 levels or lower 2:16α-OHE1 are associated with increased breast cancer risk (22, 70).

In addition to the proposed enhanced cellular proliferation due to stimulation of estrogen receptors, additional mechanisms for cancer development have also been proposed. The metabolic activity of estrogen metabolites to quinone derivatives is suggested to result in DNA changes due to oxidative damage (70).

4.2 Association between estrogen metabolites and breast cancer

Pre-clinical and clinical studies demonstrate that a greater extent of metabolism via the 16α-hydroxylation pathway is associated with breast tumours (17-24). However, these findings have not been universal (133-135).

4.2.1 Animal Studies on 2-OHE1 and 16α-OHE1

A greater extent of estrogen metabolism via the 16α-hydroxylation pathway has been shown in mice that are prone to mammary tumour development, and this up-regulation of the 16α-hydroxylation pathway is also noted well before tumour appearance (18, 19). Comparison of normal mammary epithelial cells from mice to cells that have been transformed with the *ras* oncogene, show a significant increase in 16-hydroxylation (136).

The effects of estrogen metabolites on various estrogen sensitive tissues in ovariectomized rats have been investigated (130, 137, 138). Exposure to 2-OHE1 shows no significant effects, while the effect of 16α-OHE1 on bone is that of a full estrogen agonist, and a partial estrogen agonist on the mammary gland and uterus.

4.2.2 Case-Control Studies on 2-OHE1 and 16α-OHE1

Eight case control studies have compared levels of 2-OHE1 and 16α-OHE1 in women with breast cancer to that of controls (Table 1). Six studies (17, 20, 21, 24, 139, 140) point to an increased risk of breast cancer associated with 2-OHE1, 16α-OHE1, or 2:16α-OHE1, while two found no association (60, 135). Varying methodologies were used to compare cases and controls.

The effect of the treatments used for breast cancer may impact estrogen metabolites, and in turn may have an impact of the findings on case control studies. In order to avoid this, Ho et al. (139) obtained urinary samples prior to surgery and found lower 2-OHE1 and 2:16α-OHE1, and higher 16α-OHE1 levels in cases compared to controls. Cauley et al. (60) also compared pre treatment serum samples of 272 cases to 291 controls and found that 2-OHE1 and 16α-OHE1 were 3% and 4% higher, respectively, in cases when metabolites were adjusted for age and BMI. However, there was no difference in 2:16α-OHE1, and the authors concluded that the results did not support an association between metabolites and breast cancer risk.

At the time of surgery, Osborne et al.(17) showed significantly greater 16α-hydroxylation in tissue samples from the mammary terminal duct lobular units of women undergoing mastectomy for breast cancer compared to those of women undergoing breast reduction surgery.

Schneider et al. (24) sampled women prior to the start of adjuvant treatment (i.e. chemotherapy or radiation) and found greater 16α-hydroxylation in cases compared to controls. Ursin et al. (135) compared women who had never received chemotherapy as part of treatment for breast cancer to matched controls, and found no association between risk of developing breast cancer and 2-OHE1 or 16α-OHE1 levels. Kabat et al. (20) found that women with postmenopausal breast cancer, the majority of whom had not started adjuvant treatment, had a lower 2:16α-OHE1 than controls, but there was no significant association between 2:16α-OHE1 and breast cancer risk overall. However, a low 2:16α-OHE1 was also suggestive of poorer prognosis, which could point to the ratio as a marker of progression or perhaps aggressiveness of the disease. Fowke et al. (21) sampled matched case control pairs of Chinese women, some of who had already undergone surgery and possibly adjuvant treatment. When sampling occurred prior to any treatment, a lower 2:16-OHE1 was associated with an increased risk of pre-menopausal and post-menopausal breast cancer diagnosis. However, if urine samples were collected after initiation of treatment, a higher 2:16α-OHE1 was significantly associated with breast cancer risk.

Overall, six of eight studies found a lower 2:16α-OHE1 or higher 16α-OHE1 levels in women with breast cancer compared to controls (Level of Evidence 3b and 4, Grade B and C). In addition, timing of obtaining samples may be an important consideration in evaluating case control data. Three studies were given a lower evidence rating (Level of Evidence 4) primarily due to either low sample size or incomplete reporting of subject characteristics. Of the five studies given a higher evidence rating (Level of Evidence 3b), three found a decrease risk for women with higher 2:16α-OHE for both pre- and postmenopausal women (20, 21, 139) and two found no association between 2:16α-OHE1 and breast cancer risk (both included postmenopausal women only) (60, 135). Of the three studies which received a lower evidence rating (Level of Evidence 4), one found an increased risk of breast cancer in premenopausal women who had higher levels of C16α-hydroxylation (17) and two found an increased risk of breast cancer with lower 2:16α-OHE1 in pre- and postmenopausal women (24, 140).

4.2.3 Prospective Cohort Studies

Three prospective studies have looked at 2-OHE1 and 16α-OHE1 in relation to breast cancer risk.

The Guernsey III cohort (22) followed over 5,000 women for an average of 16 years. The 146 breast cancer cases were matched to controls in a 1:3 ratio. The premenopausal cases were more likely to be nulliparous, older at age of first full-term pregnancy (FTP), and have a family history of breast cancer compared to controls. Postmenopausal cases had higher BMI than controls. The study reported a 30% reduction in breast cancer risk for participants in the highest 2:16α-OHE1 tertile compared to those in the lowest, however, this association was not statistically significant for either premenopausal (OR 0.75; 95% CI 0.35-1.62) or postmenopausal (OR 0.71; 95% CI 0.29-1.75) women.

The ORDET study (23) followed over 10,000 women for an average of 5.5 years. The 144 breast cancer cases were each matched to four controls. The premenopausal cases had a higher BMI, higher waist-hip ratio (WHR), earlier age at menarche, later first FTP, and more likely to be nulliparous than controls, while postmenopausal cases had fewer children, were older at menopause, and had a lower BMI and WHR, than controls. For premenopausal women those with a higher 2:16α-OHE1 had a reduced risk of breast cancer (OR 0.59; 95% CI 0.26-1.36) that was not statistically significant. For postmenopausal women, the association was less clear with varying increased or decreased breast cancer risk across quintiles of 2:16α-OHE1 and the association was not statistically significant.

The Danish "Diet, Cancer and Health" study followed 24,697 postmenopausal women for up to seven years (141). The 426 breast cancer cases were then matched to an equal number of controls. Cases had higher alcohol consumption, more years of education, and longer duration of hormone replacement therapy (HRT) use than controls, but none of these factors were significantly associated with breast cancer. Participants were divided into those using HRT and those not using HRT. A higher risk of estrogen receptor-positive tumour was associated with higher 2-OHE1 levels in HRT users only (incident rate ratio or IRR 1.30; 95% CI 1.02-1.66). No association with 2-OHE1 was seen with non-HRT users or for estrogen receptor-negative tumour and no association between 16α-OHE1 breast cancer was seen for all participants.

Overall, the evidence suggests a non-significant reduced risk of breast cancer in women with higher levels of 2:16α-OHE, especially for premenopausal women (Level of Evidence 2b; Grade B). In addition, HRT use may impact the association between estrogen metabolites and breast cancer risk (Level of Evidence 2b, Grade B). Based on the WCRF ratings, the link between estrogen metabolites and breast cancer risk rates as "possible". Epidemiological studies, including both case-control and prospective cohort studies, are generally supportive, but limited in number and quality, while biological plausibility has been established via *in vitro* and animal models.

4.3 Association between estrogen metabolites and other cancers

A link with other estrogen-related cancers and estrogen metabolism has also been proposed, with higher levels of 16α-OHE1 linked to endometrial cancer (142) and suggested associations with ovarian cancer (143). This is consistent with the hypothesized mechanisms, namely the estrogenic effects of 16α-OHE1, and development of estrogen-dependent cancers. In addition, a link to other cancers has also been noted, namely cervical (132, 144), prostate (145), head and neck (146), and thyroid (147). Lower levels of 2-OHE1, higher levels of 16α-OHE1 or a lower 2:16α-OHE1 were associated with an increase cancer risk in the majority of studies (132, 144-146), whereas Lee et al. (147) found higher levels of 2-OHE1 in individuals with thyroid cancer pre-operatively. In all cases, estrogen metabolites are suggested to promote cell proliferation and cell growth by binding to estrogen receptors and to act as a mitogen for the initiation of cancer (72).

Recently, polymorphisms in estrogen-metabolizing genes have been investigated in terms of cancer risk, with a positive association between CYP1A1 and hepatocellular cancer (148), and CYP1A2 and testicular cancer (149) being shown. However, no association between CYP1B1 and endometrial cancer was evident (150).

Overall, the link between estrogen metabolism and other cancers is based on a limited number of studies. Further research is needed to establish the scope of influence that estrogen metabolism may exert on overall cancer etiology. However, the current literature of breast cancer and other cancers provides evidence of the role of estrogen metabolites in cancer etiology. If physical activity is shown to alter estrogen metabolites, physical activity may play a role in prevention of a variety of cancer sites, beyond just breast cancer.

4.4 Association between estrogen metabolites and other health conditions

The effects of estrogen have been linked to numerous other health conditions and are suggested to account for the difference in disease risk between men and women, for conditions such as cardiovascular disease and osteoporosis (151). The proposed estrogenic properties of estrogen metabolites are thought to mirror the effects of estrogens themselves in terms of these various health conditions.

Higher levels of estrogens play an important role in bone health and osteoporosis risk, while accordingly an inverse relationship between osteoporosis and breast cancer risk has been reported (152-156). In terms of measures of bone growth, 2-OHE1 has little estrogenic or an anti-estrogen effect, whereas 16α-OHE1 has been shown to act as an estrogen agonist on bone in animal and *in vitro* studies (130, 138, 157). In 59 postmenopausal Korean women, 16α-OHE1 was positively correlated with spinal bone mineral density (BMD), while 2:16α-OHE1 was inversely correlated with femoral neck BMD (158). A larger study by Napoli et al. (159) did not find an association between either 16α-OHE1 or 2:16α-OHE1 and spinal BMD in a study of 170 postmenopausal women. However, Napoli et al. (160) have recently shown that women with a family history of osteoporosis have a higher 2:16α-OHE1, and significantly lower BMD at the femoral, trochanteric, and intertrochanteric regions.

Similarly, estrogen is thought to be "cardioprotective", with the incidence of coronary heart disease lower in women than men (161). Estrogenic and non-estrogen effects of estrogen metabolites, particularly 2-OHE1 and its metabolites, have been shown to have pro- and anti-oxidative effects (162-164), impact vascular tone (164), inhibit pathological cardiac fibroblast growth (164), reduce low-density lipoprotein oxidation (165), and reduce total cholesterol levels (165). However, Kuller et al. (151) found no relationship between 2-OHE1 or 16α-OHE1 and aortic calcification or carotid plaque in postmenopausal women using and not using hormone replacement therapy.

Estrogen metabolites have also been linked to other conditions, such as rheumatoid arthritis (increased 16α-OHE1) (166), lupus (increased 16α-OHE1) (166, 167) and recurrent respiratory papillomatosis (increased 16α-OHE1) (168). Further research into the association between estrogen metabolites and other conditions, as well as polymorphisms of estrogen-metabolizing genes, is needed to further understand the full health impact of estrogen metabolites, since the majority of findings are limited to single studies. At this time, the overall link between these metabolites and other disease seems to follow the pattern of risk associated with levels of estrogens. For example, while a higher 2:16α-OHE1 level may be beneficial for lowering breast cancer risk, this may increase the risk of developing osteoporosis.

4.5 Factors associated with 2-OHE1 and 16α-OHE1 levels

Estrogen metabolism may be influenced by a number of factors. If modulation of estrogen hydroxylation pathways can be achieved, either via pharmacologic methods or lifestyle factors, this may have a potential positive impact on breast cancer risk and other cancers or conditions that have been linked to estrogen metabolites (130). The effects of diet (169-173), smoking (174, 175), ethnicity (133, 176-179), family history (170), exogenous hormones (180, 181), body mass (55, 182-184), mammographic parenchymal pattern (185) and physical activity (183, 184, 186-195) have been investigated.

4.5.1 Diet

Several dietary factors have been associated with estrogen metabolism, namely dietary fat, soy products, omega3-fatty acids, and *Brassica* vegetables (e.g. broccoli, spinach, cabbage, cauliflower, Brussels sprouts, and kale).

Fowke at al. (196) investigated the impact of a variety of dietary factors on 2-OHE1 and 16α-OHE1 in 37 postmenopausal women with six 24-hour diet-recalls. Higher dietary fat-to-fibre ratio and saturated fat-to-soluble fibre ratio were associated with lower 2:16α-OHE1. Each 10g intake of saturated fat per day was associated with a 0.52 decrease in 2:16α-OHE1. An intervention study by Longscope et al. (173) looked at the effect of a low fat diet on estrogen metabolites. Six premenopausal women with normal body mass, were followed while eating their usual Western-style high fat diet (35-40% fat) for two months and then a prepared low fat diet (25% fat) for two months. A decrease in 16α-OHE1 was seen with the low fat diet. Pasagian-Macaulay (55) found no association between dietary fat reduction and 2-OHE1 or 16α-OHE1 in a nested cohort of premenopausal women undergoing a lower fat (25% fat) dietary intervention.

Soy intake and a vegetarian diet have also been linked to changes in estrogen metabolism. Adlercreutz et al. (134) compared 10 premenopausal women with breast cancer who consumed a normal omnivorous Finnish diet, to 12 women without cancer who consumed the same diet, and 11 lactovegetarian women without cancer. No significant difference in 2-OHE1 or 16α-OHE1 was found. Brown et al. (197) and Lu et al. (169) looked at the effect of an intervention of soy protein and soy milk consumption, respectively, in small intervention studies in premenopausal women. Lu (169) found an increase in 2-OHE1, no change in 16α-OHE1, and a higher 2:16α-OHE1, while Brown (197) found no effect.

The impact of omega-3-fatty acids have also been investigated in two randomized controlled trials (170, 198). Osborne et al. (170) noted a decrease in16α-hydroxylation in the group consuming omega-3-fatty acids. Haggans et al. (198) found a higher 2:16α-OHE1 with flax seed supplementation compared to those on the control cycle. Flax seeds contain omega-3-fatty acids. Brooks et al. (199) compared the effects of supplementation with soy flour to supplementation with flax seeds in postmenopausal women and showed an increase in 2-OHE1 and 2:16α-OHE1, with no change in 16α-OHE1 with flax seed supplementation. No change was noted with soy supplementation or in the placebo control group.

Brassica vegetables, such as Brussels sprouts, broccoli, cabbage, kale, turnips, collards, and cauliflower, are broken down into dietary indoles. Administration of indole-3-carbinol to rats shows increased hepatic oxidation of estrogen (200) and a 15% increase in 2-hydroxylation (201). Oral administration of indole-3-carbinol in humans shows a similar increase in 2-hyroxylation (171) and increase in the excretion of 2-OHE1 (202). Fowke et al. (172) looked at *Brassica* consumption in 34 postmenopausal women. The participants were instructed in ways to increase consumption of appropriate vegetables. While crude 2:16α-OHE1 did not change from baseline, adjustment for other dietary parameters showed an association between *Brassica* vegetables intake and an increase in 2:16α-OHE1. In fact, for each 10 gram per day increase in *Brassica* consumption, there was an increase in the ratio of 0.08.

4.5.2 Smoking

Smoking is suggested to lower estrogenic activity and has been linked to lower risk of endometrial cancer (203), earlier natural menopause, and increase in osteoporosis (204-206). Decreased synthesis or increased hepatic metabolism of estrogen have both been proposed as mechanisms (175). Michnovicz et al. (175) compared 2-OHE1 levels in 14 smokers (at least 15 cigarettes per day) to 13 non-smokers in terms of 2-OHE1 levels. All were healthy premenopausal women with BMI < 25, who were not using oral contraceptives. Higher 2-OHE1 was found in the smokers. A similar subsequent study by the same authors also found higher 2-OHE1 levels in smokers compared to non-smokers (174).

4.5.3 Ethnicity

The impact of ethnicity on estrogen metabolism has been investigated as a possible explanation of differing breast cancer incidence rates in different ethnic groups. In North America, African Americans have the highest rates of premenopausal breast cancer and Asians have lower breast cancer rates across all age groups (99). Ursin et al. (176) compared 67 postmenopausal Chinese women living in Singapore to 58 postmenopausal women living in the United States, who were predominantly African-American. Higher 2-OHE1 was noted in Singapore Chinese women, while no statistical difference in 16α-OHE1 or 2:16α-OHE1 was found. Higher levels of E1, E2, E3, seen in the women in the United States, were suggested to be more important factors in the difference in breast cancer risk between the two populations. Taioli et al. (178) found a significantly lower 2:16α-OHE1 in 18 African-American women, compared to 15 Caucasian women, who ranged in age from 18-73 years, and therefore included both pre-

and postmenopausal women. Coker et al. (177) compared 74 women with breast cancer to 58 controls and found lower 2-OHE1 levels in African American cases compared to Caucasian cases, for both pre- and postmenopausal women. In addition, African American women (cases and controls combined) had lower 2-OHE1 levels than Caucasian women (combined cases and controls). Adlercreutz et al. (133) compared premenopausal Asian women, who were recent immigrants to Hawaii, to Caucasian women living in Finland. The Asian women had higher 16α-hydroxylation, while 2:16α-OHE1 was higher in the Finnish women.

A recent comparison of Asian American women, who were born in the "East" (e.g. China, Taiwan, Hong Kong, Macao, Japan, the Philippines, countries in Southeast Asia and the Malaysian Peninsula, Singapore, India and countries in the southwest Pacific Ocean, except Australia and New Zealand) and migrated to the United States (e.g. San Francisco-Oakland area, Los Angeles and Oahu, Hawaii), to Asian American women who had been born in the "West" (e.g. North American, western or central Europe, the former USSR, Australia and New Zealand) and lived in the United States, and found that 2:16α-OHE1 was 20% lower in premenopausal women born in the "West" (179). In postmenopausal women 2:16α-OHE1 was 23% lower in those born in the "West", but this finding was not statistically significant. Length of time living in the United States did not impact 2:16α-OHE1.

4.5.4 Exogenous hormones

The effect of hormone replacement therapy (HRT) or oral contraceptive (OC) use on estrogen metabolism was investigated by Mueck et al. (180). Fifty-five postmenopausal women were randomly given one of two HRT regimes, while 63 premenopausal women were randomly given one of two OC regimes. Overnight urine was collected and metabolites were determined by enzyme immunoassay. Levels of 2-OHE1 and 16α-OHE1 were significantly higher after HRT administration, but there was no statistically significant change in 2:16α-OHE1. In the OC groups, a trend towards higher 2-OHE1 was seen with both OC regimes, and a decrease in 16α-OHE1 and 2:16α-OHE1 was seen with the use of ethinylestradiol plus dienogest, but not with ethinylestradiol and norethisterone acetate.

Jernstrom et al. (181) examined a number of suspected breast cancer risk factors and factors that have been associated with estrogen metabolites in 513 premenopausal women. Women who used OC had a lower median 2:16α-OHE1 than non-OC users. Overall, the strongest predictor of 2:16α-OHE1 was OC use. In non-OC users, coffee consumption, Asian ethnicity, and higher BMI were associated with lower 2:16α-OHE1. The effect of HRT on 2-OHE1 and 16α-OHE1 levels in breast cases and controls was investigated in the Danish "Diet, Cancer and Health" cohort study (141). No difference in 16α-OHE1 levels was found, while higher 2-OHE1 was associated with a higher risk of estrogen receptor-positive breast cancer in HRT users only.

4.5.5 Body Composition

An association between body composition (i.e. body mass, BMI, WHR or adiposity) and estrogen metabolism has been noted in some (182, 184, 207), but not all studies (55, 181, 183). Fishman et al. (182) compared seven women with anorexia nervosa to four obese (75% above normal body mass) and eight normal body mass controls, in terms of estradiol, estrone, 2-OHE1, and body mass. The amount of 2-OHE1, as measured by three-day urinary excretion of intravenously administered labelled estradiol, was significantly higher in individuals with anorexia nervosa compared to both control and obese participants. In addition, normal body mass controls had significantly higher levels of 2-OHE1 compared to the obese group. The author attributed the difference in 2-OHE1 to body mass, body composition, or nutrition. Schneider et al. (207) showed that obesity (> 60% above ideal body mass) was associated with a significant decrease in hydroxylation at C-2 (producing 2-OHE1 and 4-OHE1), while hydroxylation at 16α-site was unchanged compared to normal body mass controls, using intravenously administered labelled estradiol. A cross-sectional study by Matthews et al. (184) in Caucasian women living in the United States and Asian women living in China found a lower 2:16α-OHE1 in women with a higher BMI ($\geq 25\ \mathrm{kg \cdot m^{-2}}$) and lower self-reported levels of physical activity. However, women who were active but who also had a similarly high BMI did not have a reduction in 2:16α-OHE1. Body composition has also been suggested to be a key determinant of estrogen metabolism in other studies looking at the impact of physical activity on estrogen metabolism, with higher 2-OHE1 levels noted in leaner athletes (187, 188, 208). Contrary to these noted associations, Jernstrom et al. (181) found no association between 2-OHE1, 16α-OHE1 or 2:16α-OHE1 and height, body mass or BMI in a cross-sectional study of premenopausal women.

The effect of body mass loss on estrogen metabolites was assessed in a nested cohort who undertook a 20-week lower fat (25% fat) dietary regime. The intervention group lost an average of 4.5 kg and this was not associated with a change in 2-OHE1 or 16α-OHE1 (55). However, an increase in body mass, BMI, and WHR in the control group was associated with a higher 2:16α-OHE1, which is inconsistent with the previous literature (182, 184, 207).

No association between baseline BMI, body fat, lean body mass, abdominal fat, or subcutaneous fat and estrogen metabolites was noted in 170 postmenopausal women prior to the start of a year-long aerobic physical activity intervention. However, post-intervention, a weak positive association between an increase in lean body mass and an increase in 2-OHE1 was seen in the exercise group, while a weak inverse relationship between intraabdominal fat and 2-OHE1 was seen in the control group (183).

4.5.6 Family History

Osborne et al. (170) compared 70 premenopausal women who had a first degree family history of breast cancer to 27 women who did not have this history. Women without a family history of breast cancer had higher 2-OHE1 and 16α-OHE1 levels, but the 2:16α-OHE1 was similar between the two groups. Jernstrom et al. (181) also found no association between family history of first or second degree relatives with breast cancer and estrogen metabolites.

4.5.7 Mammographic Parenchymal Patterns

Mammographic density is an established risk factor for breast cancer (209), and is used as an intermediate marker of breast cancer in epidemiology studies (210). A study nested within a larger cross-sectional study matched 70 postmenopausal women with low mammographic density (N1) to 70 women with high mammographic density (P2/DY), on age, and years since menopause. Women with the P2/DY pattern had higher 2-OHE1, 16α-OHE1 and 2:16α-OHE1 (185). These findings point to an increase in breast cancer risk, rather than a decrease, associated with higher 2:16α-OHE1.

5.0 Association between physical activity and reproductive function

Menstrual dysfunction associated with endurance sports first received attention in the late 1970's and early 1980's (211), and early observational research attempted to establish the extent of amenorrhea in athletic populations (primarily runners) (212, 213). This was followed by cross-sectional studies linking menstrual dysfunction, primarily amenorrhea, to decreased bone mineral density (214) and altered sex steroid hormone levels (215) in female athletes. Research in the late 1980's and early 1990's began to focus more on the proposed mechanisms of menstrual dysfunction, such as body mass, body composition, intensity and duration exercise, stress, pre- or post-menarcheal commencement of training, and diet (216). While exercise has been linked to a continuum of menstrual disturbances ranging from luteal phase defects (LPD) to amenorrhea, the mechanism is not fully understood (217, 218). To this point, exercise training has been viewed as a single stress or stressors that impinge(s) on the functioning of the gonadotrophin-releasing hormone (GnRH) pulse generator in the hypothalamus of the brain, which in turn leads to alterations in luteinizing hormone secretion (LH), and finally to reduced support to the ovary by gonadotrophins (218). A review by Loucks (219) outlines the prominent recent theories on disruption of the GnRH pulse generator and the resulting alterations in reproductive function in women: 1) critical body fat levels, 2) exercise stress, 3) energy availability. Recent research on this topic suggests that exercise, or other stressors, have no disruptive effect on reproductive function beyond that of their energy cost on energy availability, and reinforces the importance of energy availability as the route by which exercise exerts an effect on reproductive function (219, 220).

Energy availability can be reduced by lowering energy intake alone, increasing energy expenditure alone, or a combination of the two (219). This theory of disruption in reproductive function secondary to energy availability is supported by both human (221) and animals studies (222). Loucks and Thuma (221) have demonstrated altered LH pulsatility in sedentary young women with an acute exercise test under strict energy intake and expenditure control, at a specific threshold (< 30 kcal/kg LBM/d) (221). In primates, LH pulsatility and menstrual dysfunction has been induced in a number of months by a progressive daily treadmill running program, while dietary intake was maintained at stable levels (222). Rapid recovery of reproductive hormones occurred with supplemental calories while training was maintained (223).

The alterations in reproductive function may have a positive effect on reducing overall breast cancer risk. In addition, effecting energy availability by increasing energy expenditure through physical activity or a combination of reduced dietary intake and increased energy expenditure, would appear to be more feasible on a population level than severe dietary restriction. Westerlind (224) also points out that numerous physiological and biochemical changes occur in response to exercise, than do not occur with energy restriction. Therefore, physical activity has the potential to impact not only global reproductive function, but also may cause more subtle changes in the hormonal milieu, such as estrogen metabolism, which may have an impact on cancer risk.

5.1 Changes in sex steroids hormones and gonadotrophins due to physical activity

Comprehensive reviews by DeCree (225) and Consitt (226) outline the effects of physical activity on female sex steroid hormones in premenopausal women.

5.1.1 Acute Exercise

Short-term increases in estradiol levels are seen with acute aerobic exercise, and appear to be dependent on intensity of the exercise and phase of the menstrual cycle, with higher levels noted in the luteal phase (226). Progesterone levels have been found to increase with incremental exercise during only the luteal phase, or not change during exercise (225). Testosterone levels have been shown to rise linearly with both exercise intensity and duration, and return to resting levels within hours of the exercise bout (226). The increased levels of sex steroid hormones have been attributed to slower degradation due to reduced hepatic blood flow seen with acute exercise, rather than increased production (226).

The reported effect of acute aerobic activity on gonadotrophins, namely LH and follicle-stimulating hormone (FSH), is variable and a comprehensive review on this topic was not found. In eumenorrhoeic athletes, a decrease in LH pulse frequency (227) and/or an increase in pulse amplitude (228) have been shown. In eumenorrheic sedentary women, Williams et al. (218) found no effect of an acute bout of moderate intensity activity on LH pulse frequency or mean serum LH concentration. However, in a similar population, several days of strenuous exercise has resulted in slowing of LH pulsatility, but only if caloric restriction was included (229, 230). FSH levels remained the same with a bout of treadmill running at 60-80% of VO_2max for 75 minutes in eumenorrheic trained marathon runners, while stationary cycling at the same workloads in eumenorrheic untrained women, caused FSH to decrease significantly, but more so in the luteal compared to the follicular phase (231). This is similar to the findings of Bonen et al. (232) who found no change in FSH with acute exercise.

5.1.2 Chronic Exercise

Chronic aerobic exercise in normally cyclic premenopausal women lowers resting levels of estradiol, progesterone, and testosterone, and increases levels of SHBG (225, 226). Lower levels of progesterone and estradiol are found, especially in the luteal phase of the menstrual cycle (225). Overall, decreases in LH and FSH are associated with menstrual dysfunction, however, there is some evidence to suggest that moderate training may cause smaller reductions in gonadotrophins (215). A review by De Souza (217) on menstrual dysfunction reported that chronic aerobic exercise training has also been associated with luteal phase dysfunction and amenorrhea.

In an observational study, which collected daily urine samples over three consecutive menstrual cycles in sedentary and recreationally active women (running 32.4±3.5 kilometres per week, at an average heart rate of 132.2±4.9 beats per minute), it was shown that in the active women, 45% were ovulatory, 43% had LPD, and 12% were anovulatory (50), while none experienced amenorrhea. Active women, regardless of ovulatory status, had lower luteal progesterone levels compared to the sedentary women, and lower estradiol levels in the early follicular phase (day 2-5). This decrease in follicular estradiol excretion may be a result of blunted FSH elevation during the luteal-follicular transition, and may be indicative of LPD (50).

5.1.3 Intervention studies

While observational research points to the presence of a continuum of menstrual dysfunction (amenorrhea, anovular cycles and luteal phase deficiency) in athletes, and lower progesterone and estradiol levels in eumenorrheic athletes compared to controls

(50, 233-238), only five intervention studies have looked at aerobic training and reproductive function in untrained women (Table 2).

Two intervention studies are uncontrolled trials (239, 240) and three studies are randomized controlled trials that assign women to different conditions, a weight maintenance group or weight loss group (241), three ranges of training mileage (<10, 10-20 and 20-30) for either two or four months (242), or one of two exercise intensities for one year (243).

Overall, two studies found that a moderate intensity running intervention did not disrupt reproductive function (242, 243), two studies found minor changes in measures of reproductive function (239, 240), while another, which had significantly greater exercise intensity and volume, induced menstrual dysfunction (241). In general, these studies suffered from methodological problems around sample size, maintenance of group assignment for analysis (242), and non-equivalent controls (243, 244).

In general, the literature suggest that exercise may cause minor shifts in the hormonal milieu of premenopausal women but a program of significant intensity and volume is needed to induce menstrual dysfunction (Level of Evidence 3, Grade C).

5.2 Changes in estrogen metabolites due to physical activity

To date there are five prospective observational studies (184, 186-189) (Table 3), four intervention studies using acute bouts of exercise (191, 193-195) and two chronic exercise intervention studies (55, 183) that have investigated the impact of physical activity and estrogen metabolites, 2-OHE1 and 16α-OHE1.

5.2.1 Prospective observational studies

An initial interest in this area in the 1980's resulted in three studies. Over a two year period, Russell et al. (186) followed competitive female swimmers and identified five who developed oligomennorrhea with training. The estrogen metabolites levels in these five competitive swimmers (mean age of 14.4 years) were compared to their own samples during various training phases of the year and to six controls who did not participate in "any organized physical activity" (mean age of 19 years). Estrogen metabolites were measured in serum using a radioimmunoassay (RIA). During "moderate" intensity training, levels of 2-OHE1 were similar between swimmers and controls ($p=.08$). However, during the "strenuous" training period, the swimmers had significantly higher levels of 2-OHE1 compared to controls ($p<0.001$). "Strenuous" training also coincided with the development of menstrual dysfunction.

Russell et al. (187) then compared competitive swimmers with menstrual dysfunction (mean age 14.8 years), to runners (25-30 miles per week) who had normal menstrual cycles (mean age 30.9 years), and sedentary controls who had regular menstrual cycles and similar height, body mass, and percent body fat to the other two groups (mean age 27.9 years). Estrogen metabolites were measured using a RIA kit for serum. Slightly higher 2-OHE1 levels were seen in the competitive swimmers compared to that of the runners (p= not reported), and both groups were higher than sedentary controls ($p<0.01$). In addition, comparison pre- and post-exercise bout samples showed higher 2-OHE1 values following an acute exercise bout.

Snow et al. (188) followed varsity rowers over a nine-month training season, from three months of low intensity, to three months of high intensity and another three months of low intensity training. Five rowers who developed menstrual dysfunction during the high intensity training were compared to five rowers who did not, and to four "non-athletic" controls. Estrogen metabolism was measured using administered labelled tracers. The rowers and controls were all similar in age (22-23 years), height, and body mass. There was no difference in 16α-OHE1 between the three groups, however, the rowers who developed menstrual dysfunction had higher 2-hydroxylation than the other rowers (p=.01) and controls (p=.05). Other rowers and controls did not differ (p=not reported). Contrary to Russell et al. (186), the pattern of metabolism was consistent for each group across the training phases. Body mass change, and percent body fat changes were similar in all rowers across the training. However, the leaner the rower, the greater the extent of 2-hyroxylation (r=.55; p=.027).

More recently, Bentz et al. (189) looked at a sample of 77 women with normal menstrual cycles (mean age 26 years), who were asked to keep a physical activity log, which included household, work, leisure, exercise and sports related physical activity, for two weeks. Participants then provided a urine sample in the luteal phase of the menstrual cycle that was analyzed using a solid-phase enzyme immunoassay. Controlling for age and BMI, a higher 2-OHE1 and 2:16α-OHE1 was shown with higher reported MET-hours per day (p=.018 and p=.020, respectively). Between the highest and lowest quartiles of 2:16α-OHE1 and 2-OHE1, frequency of physical activity was also significantly different (p=.023 and p=.045, respectively). However, when divided into normal body mass (BMI 17.8 – 25.9) and overweight (BMI 25.1-29.4), a relationship with MET-hours of physical activity was no longer significant for the normal body mass group, but remained significant for the overweight group. It was found that more than half of the "overweight" group reported "highly competitive" or "above average" levels of fitness. BMI is not a very sensitive measure of body composition and the authors suggest that these individual were unlikely to have a high level of adiposity, which may have impacted their findings related to BMI (245).

Matthews et al. (184) looked at self-reported physical activity in two groups of women, one being postmenopausal women in North American (questionnaire which included household, leisure, occupational, sports, exercise or recreational activities over a 7 day period, administered twice and averaged), and the other being pre- and postmenopausal Chinese women in Shanghai (questionnaire on lifetime occupational activity and non-occupational activity in adolescence and in adulthood). Estrogen metabolites were measured by solid-phase enzyme immunoassay. Active women, those who reported greater than or equal to 0.5 MET hours per day of LTPA, had a higher 2:16α-OHE1 compared to "inactive" women who reported less LTPA . The 2:16α-OHE1 was 18% higher in North American women ($p=.32$) and 20% higher in Chinese women ($p<0.05$). However, this was non-significant for North American women and non-significant after dietary adjustment in Chinese women. The combination of physical activity and body composition was significant. A lower 2:16α-OHE1 was seen in women with higher BMI and low LTPA, compared to low LPTA counterparts with lower BMI ($p=.04$). For women with higher BMI, who were physically active, 2:16α-OHE1 remained high and was similar to those with a lower BMI who were also physically active ($p=.89$). The amount of physical activity reported by the "active" women (mean approximately 1.0-2.0 MET-h/day of LTPA), was quite low and corresponded to 15-30 minutes of walking per day.

The early studies (186-188) all suffer from a number of methodological limitations. Sample sizes were small, and the studies were generally underpowered to detect differences in estrogen metabolites. Menstrual function was not standardized, resulting in comparisons between individuals with and without menstrual dysfunction, and mature versus immature menstrual cycles. These studies also used an older version of RIA (186, 187) or administration of a labelled tracer (188) to measure estrogen metabolites, which make comparison to the newer solid-phase enzyme immunoassay (52-55) difficult.

Two recent studies (184, 189) have improved on the methodology of the earlier studies. Both used larger sample sizes, standardized menstrual status and analysis of estrogen metabolites was done using solid-phase enzyme immunoassay. However, both used BMI as a measure of adiposity, which is problematic, especially for more athletic populations (245). In addition, all studies measured physical activity with self-reported measures, such as swim distance, running mileage, participation in a varsity-rowing program, LTPA or occupational activities. Self-reported physical activity suffers from a number of methodological issues (40), in particular over reporting of frequency, duration and intensity.

5.2.2 Acute intervention studies

A series of intervention studies using short training bouts (191, 193-195) showed no change in 2-OHE1 (193) or decreased 2-OHE1 (191, 194, 195) measured in plasma using an RIA technique developed by the investigators . The training bouts consisted of five consecutive days of interval training on a cycle ergometer, once in the follicular and once in the luteal phase of one menstrual cycle. Training started with two minutes at 90% of maximum work capacity, as determined by an incremental exercise test, and two minutes recovery. This was continued until 90% could not be maintained, at which time the intervals were dropped to 80% and then 70%, until the participant reached exhaustion. The length of the training bout was usually approximately 90 minutes. All participants had regular menstrual cycles, and the number of participants in each study ranged from one to 15.

Further studies with a greater number of participants, using other methods of estrogen metabolite measurement, and validated by other investigators would add to the evidence for an acute effect of exercise on estrogen metabolites. In addition, research looking at the acute effect of exercise for a longer time course following the exercise bout would increase the applicability of these changes to the link between overall physical activity participation and breast cancer risk.

5.2.3 Chronic exercise training intervention studies

Two intervention studies (55, 183) have investigated the impact of physical activity and estrogen metabolites, 2-OHE1 and 16α-OHE1.

As part of a larger cohort study, urinary estrogen metabolite pattern of 174 premenopausal women, age 44-50 years, was measured with a solid-phase enzyme immunoassay. Urine sampling was not standardized to a specific time of the menstrual cycle. The women randomized to the invention group received a 20-week group-based lifestyle counselling program aimed at reducing dietary fat intake ($\leq$25% of total calories), increasing physical activity (i.e. daily moderate-intensity activities such as walking), and body mass loss. The intervention group increased their physical activity level by approximately 400 kilocalories per week. A significant increase in 2:16α-OHE1 was seen in both the intervention (10% increase; p=.03) and control group (12%

increase; p=0.01), but no statistically significant difference in change between groups was seen at the end of the intervention. An increase in body mass (r=.14, p=.05), BMI (r=.16, p=.04) and WHR (r=.19, p=.01) was associated with an increase in 2:16α-OHE1, however, this was limited to the control group. No relationship between body mass loss (mean loss 5.4 kg) and 2:16α-OHE1 was seen in the intervention group. A higher concentration of 2-OHE1 or 2:16α-OHE1 associated with increased body mass is contrary to other literature (184). The main limitations of this study were the use of a combined diet and exercise intervention, and the limited reporting on the physical activity aspect of the intervention and associated outcomes, making determination of the effect of physical activity on estrogen metabolites difficult.

Atkinson et al. (183) measured urinary estrogen metabolites in 170 postmenopausal women. At baseline, participants were generally overweight (BMI 24-40) and previously sedentary (< 60 min/week of exercise at a level producing sweat). Those randomized to the intervention group participated in a 12-month moderate-intensity aerobic activity program, while those randomized to the control group participated in a stretching placebo group. Adherence rates to the exercise program were reported, with 93% of women achieving at least 80% of the exercise goal of 225 minutes a week of moderate-to-vigorous exercise. No significant change in 2-OHE1 (p=.76), 16α-OHE1 (p=.64) or 2:16α-OHE1 (p=.57) was seen. Body composition was assessed by dual-energy x-ray absorptiometry. A weak association between changes in lean body mass and 2-OHE1 was seen in the exercise group (r=.25, p=.03) and a weak association between changes in intra-abdominal fat and 2-OHE1 was seen in the controls (r= -.23, p=.05).

The study by Atkinson et al. (183) used the strongest methodology to date. This trial was a well described randomized controlled trial in a large group of homogenous women, with a well defined amount of physical activity and gold standard measure of body composition. Based on this study, 12-months of moderate-to-vigorous aerobic activity does not change estrogen metabolites in postmenopausal women (Level of Evidence 1b, Grade A).

Despite the lack of effect seen with these randomized controlled trials, two recent cross-sectional studies suggest that higher MET-hours per day of physical activity are associated with levels of 2-OHE1 and 2:16α-OHE1, however, this association may be related to issues of body composition (Level of Evidence 2b, Grade B). In addition, three older studies point to higher 2-OHE1 levels in more active young women, especially those who develop menstrual dysfunction associated with exercise (Level of Evidence 4, Grade C) and an acute effect of high intensity exercise bouts on estrogen metabolism in young women (Level of Evidence 4, Grade C). Overall, the evidence surrounding alterations in estrogen metabolism with physical activity is "insufficient" to determine an association. Results are suggestive of a possible association, with generally consistent results, but a greater number of studies using better methodology are needed to confirm this association.

6.0 Research Question

A link between estrogen metabolites, 2-OHE1 and 16α-OHE1, and breast cancer risk has been reported, and the ability of physical activity to alter the pattern of these estrogen metabolites is commonly cited. However, the published literature on physical activity and changes in 2-OHE1 and 16α-OHE1 has been limited in scope and suffered from a number of methodological problems.

The aim of this dissertation was to improve on previous research methodology used to examine the association between physical activity and estrogen metabolites. Study One aimed to improve on previous cross-sectional observational studies by using a criterion measure of habitual moderate and vigorous physical activity, namely cardiorespiratory fitness as determined by maximal aerobic capacity using indirect calorimetry (VO_2max), rather than self-reported physical activity, and a more homogenous population by limiting the study to healthy, normally cyclic, reproductively mature, premenopausal women. Based on these findings, Study Two aimed to determine the effect of a supervised 12-week intervention of moderate-to-vigorous aerobic exercise training program, similar to the public health guidelines for reducing breast cancer risk (i.e. 45 minutes or more, of moderate-to-vigorous activity on five or more days per week), on estrogen metabolites (2-OHE1 and 16α-OHE1) in healthy, normally cyclic, sedentary premenopausal women.

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Table 1 – Summary of case-control studies examining breast cancer risk and 2-OHE1 and 16α-OHE1 levels

Author	**Design**	**Participants**	**Menopausal status**	**Association**
Cauley, 2003 (60)[°]	Nested case-control from prospective cohort	Cases = 272 Controls = 291	All postmenopausal	-
Fowke, 2003 (21)[°]	Population based case-control	Cases = 101 Controls = 101	72% premenopausal	+
Ho, 1998 (139)[°]	Clinic based case-control	Cases = 65 Controls = 36	34% premenopausal	+
Kabat, 1997 (20)[°]	Clinic based case-control	Cases = 42 Controls = 64	Mixture of pre-, peri-, and postmenopausal	+ (Post menopausal only)
Osborne, 1993 (17)[ε]	Hospital based case-control	Cases = 4 Controls = 4	All premenopausal	+
Rogan, 2003 (246)[*]	Clinic based case-control	Cases = 28 Controls = 49	Mixture of pre-, peri-, and postmenopausal	-
Schneider, 1982 (24)[ε]	Case-control	Cases = 33 Controls = 10	Mix of peri- and postmenopausal	+
Ursin, 1999 (135)[°]	Population based case-control	Cases = 66 Controls = 76	All postmenopausal	–
Zheng, 1998 (140)[°]	Hospital based case-control	Case = 20 Control = 20	Not reported	+

Notes: Higher 16α-hydroxylation or lower 2: 16α-OHE1 associated with higher breast cancer risk denoted as "+"; No association denoted as "-".[°] Estramet ELISA from Immuna Care; [ε] Radio- labeled tracers; [*]High Performance Liquid Chromatography (HPLC).

Table 2 – Summary of intervention studies examining the effects of physical activity on sex-steroid hormones

	Design	N	Exercise	Estrogens	Progesterone	FSH	LH	MD
Bonen (242), 1992	Uncontrolled non-randomized trial Six groups	57	-4-5/week -2 or 4 months -75% of VO_2max -Duration not stated -Running	/	⇔	**Varied**	⇔	**N**
Bullen (239), 1984	Uncontrolled non-randomized trial One group	7	-6x/week -8 weeks -85% of HRmax -20-45 min -Cycling 2x/week; Running 4x/week	⇓ (only with MD)	⇓	/	/	**Y (4 of 7)**
Bullen (241), 1985	Uncontrolled randomized trial Two groups: Weight maintenance (WM) & weight loss (WL)	28	-5x/week -8 weeks -70-80% VO_2max for runs; "moderate" intensity of sports - Duration by mileage; Sports 3.5 h/day - Running; Recreational sports	/	/	⇓	/	**Y** **WM = 9/12** **WL = 15/16**
Keizer (240), 1987	Uncontrolled non-randomized trial One group	8	-3-4x/week -3 months -60-80% VO_2max -20-75 minutes -Running 2-3d/wk; Cycle Ergometer 1d/wk	⇓	⇔	/	/	**N**
Rogol (243), 1992	Uncontrolled randomized trial Two groups	23	4-6x/week -2 months -LT or above LT -6.25-40 miles/week -Running	/	/	⇔	/	**N**

Abbreviations: FSH, follicle stimulating hormone; LH, lutenizing hormone; VO_2max, maximal aerobic capacity.

Notes: ⇔ denotes no change; ⇓ denotes a decrease; / denotes not reported.

Table 3 – Summary of studies examining physical activity and 2-OHE1 and 16α-OHE1 levels

Author	**Design**	**Sample**	**Exercise** Method of reporting/intervention *Amount*	**Analysis** Method *Sample* (Cycle Phase)	**Findings**
Russell, 1984 (186)	Case-control	N= 5 swimmers (oligomennorehic) N= 6 controls (eumenorrehic)	Self-report/sports team training *Swim =Strenuous training (100,000 yards/week)* *Moderate training (60,000 yards/week)* *Controls = No regular PA*	RIA *Serum* (Eumenorrheic = Day 13-17)	+
Russell, 1984 (187)	Cross- sectional	N= 6 swimmers (oligomennorehic) N=7 runners (eumenorrehic) N=7 controls (eumenorrehic)	Self-reported PA level *Swim = ≥5day/week with team* *Run ≥6day/week, 25-30 miles/week* *Control = No regular PA*	RIA Serum (Eumenorrheic = Day 15)	+
Snow, 1989 (188)	Case-control	N = 5 rowers (eumenorrehic) N= 5 rowers (oligomennorehic N= 4 controls (eumennoehic)	Self-report/sports training *Rowers = Strenuous training (8-11 workouts/week) or Low Intensity training (5-6 workouts/week)* *Controls = No regular PA*	RIA *12 hour overnight urine* (Time in cycle not controlled)	+
Pasagian-Macaulay, 1996 (55)	Nested randomized controlled trial	N= 84 Intervention N=90 Control Premenopausal North American (93% White) BMI = 20-34	20 week weight loss, diet (low fat), physical activity (moderate intensity aerobic activity *↑400 kcal/week, mainly walking*	ImmunaCare ELISA *Fasted spot urine* (Time in cycle not controlled)	-

Table 3 – continued

Author	**Design**	**Sample**	**Exercise** Method of reporting/intervention *Amount*	**Analysis** Method *Sample* (Cycle Phase)	**Findings**
Matthews, 2004[#] (184)	Cross-sectional	N= 37 Post menopausal North American (not defined further) BMI = 20.5-41.8	Exercise logs (2 weeks) for occupational, household and leisure activities (Modified Baecke) *Mean MET-hours/day for LTPA = 2.1 ± 2.1 SD*	ImmunaCare ELISA *24 hour collection* (Not required)	- overall + with BMI
Matthews, 2004[#] (184)	Cross-sectional	N=109 Pre and Postmenopausal Chinese (Shanghai)	Lifetime occupational and non-occupational in adolescence and adulthood *MET-hours/day for LTPA = 0.7 ± 3.6 SD*	ImmunaCare ELISA *First morning urine* (Not reported)	- overall + with BMI
Atkinson, 2004 (183)	Randomized controlled trial	N=87 Intervention N = 86 Control Postmenopausal 86.5 % Non-Hispanic White BMI 24-40	12 month aerobic activity 45 mins; 5 day/week 60-70% max. HR (from VO_2max) *93% met goal (225 minute/week of moderate-vigorous exercise; 11.7% increase in VO_2max)*	ImmunaCare ELISA *Fasting spot urine* (Not required)	-
Bentz, 2005 (189)	Cross-sectional	N=77 Premenopausal North American (not African American) BMI = 18-30	Exercise log (2 weeks) *Mean MET-hours/day =3.5 ± 0.3* *Mean frequency/week = 5.0 ± 0.2*	ImmunaCare ELISA *First morning urine* (Day 5 prior to onset of menses)	+

Abbreviations: LTPA, leisure time physical activity; kcal, kilocalories; MET, metabolic equivalent.

Notes: Higher 2-OHE1 or higher 2:16α-OHE1 associated with physical activity denoted as "+"; No association denoted as "-";

[#] Published as one paper

CHAPTER 3 – Study One

Associations between Aerobic Fitness and Estrogen Metabolites in Premenopausal Women.

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Associations between Aerobic Fitness and Estrogen Metabolites in Premenopausal Women

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ABSTRACT

CAMPBELL, K. L., K. C. WESTERLIND, V. J. HARBER, C. M. FRIEDENREICH, and K. S. COURNEYA. Associations between Aerobic Fitness and Estrogen Metabolites in Premenopausal Women. *Med. Sci. Sports Exerc.*, Vol. 37, No. 4, pp. 585–592, 2005. Chronic physical activity may alter estrogen metabolism, a proposed biomarker of breast cancer risk, by causing a shift toward higher 2-OHE1 and lower 16α-OHE1 levels. **Purpose:** To investigate the association between an objective indicator of chronic exercise, aerobic fitness, and 2-OHE1 and 16α-OHE1 in premenopausal women. **Methods:** Women with high aerobic fitness ($N = 17$; $\dot{V}O_{2max} \geq 48\ mL \cdot kg \cdot min^{-1}$) were compared with women with average aerobic fitness ($N = 13$; $\dot{V}O_{2max} \leq 40\ mL \cdot kg \cdot min^{-1}$) in terms of 2-OHE1 and 16α-OHE1 profiles. Participants were healthy, regularly menstruating, Caucasian women, aged 20–42 yr, with a normal body mass index (BMI) of 18–24, not using pharmacologic contraceptives. We measured height, weight, sum of four skinfolds, and maximal aerobic fitness ($\dot{V}O_{2max}$), using an incremental cycle ergometer test. Urine samples were collected during the follicular and luteal phase of the menstrual cycle. **Results:** There were no statistically significant differences between average and highly fit women for 2-OHE1, 16α-OHE1, or the 2:16α-OHE1 ratio in either the follicular or luteal phase. However, the high-fitness group showed a trend toward a higher luteal 2:16α-OHE1 ($P = 0.20$). In ancillary analyses, a higher sum of skinfolds was associated with significantly higher luteal 16-OHE1 levels ($r = 0.39$, $P = 0.03$) and lower luteal phase 2:16 OHE ratio ($r = -0.41$, $P = 0.02$). Higher BMI was associated with lower follicular phase 2-OHE1 ($r = -0.37$, $P = 0.04$) and lower follicular 2:16 OHE1 ratio ($r = -40$, $P = 0.03$). **Conclusion:** This exploratory study is the first to investigate the association between aerobic fitness and estrogen metabolites in premenopausal women using metabolic parameters. We observed no statistically significant association between aerobic fitness and 2-OHE1 and 16α-OHE1, but found that body composition was associated with 2-OHE1 and 16α-OHE1 levels. **Key Words:** CARDIORESPIRATORY FITNESS, PHYSICAL ACTIVITY, BREAST CANCER, BIOMARKER

Epidemiologic data suggest a 30–40% decrease risk of breast cancer in women who are physically active (15). However, the underlying biologic mechanisms mediating the association between physical activity and breast cancer risk have not been established. Possible factors include changes in endogenous sex-steroid hormone production, estrogen metabolism, circulating concentrations of peptide hormones and growth factors, obesity, central adiposity, and immune function (15).

Cumulative lifetime exposure to sex-steroid hormones, particularly estrogen, is thought to play an important role in breast cancer risk (6). Physical activity is suggested to reduce breast cancer risk by lowering resting levels of estradiol and progesterone, and increasing levels of sex hormone binding globulin (15). However, a significant reduction in sex steroid levels due to physical activity may be related to a continuum of menstrual dysfunction (11). The level of activity associated with a reduction in breast cancer risk (i.e., moderate-intensity leisure-time activity for at least 4 h·wk^{-1}) is unlikely to result in a significant decrease in hormone levels or in menstrual dysfunction (15). It is possible, however, that moderate levels of physical activity may have more subtle effects on sex-steroid hormone levels such as altering estrogen metabolism.

Estrogen metabolites, which result from the hydroxylation of the parent estrogens (estradiol and estrone), have been implicated as a subtle link between estrogen and its proposed biologic effects, especially in health conditions associated with estrogen levels (e.g., breast cancer, reproductive cancers, osteoporosis) (23,33). Modulation of estrogen metabolism through lifestyle factors, such as diet (14), weight loss (13,24,30), smoking (22), and physical activity (1,20,26,27,30), has been investigated, and may explain the association between lifestyle and reduced breast cancer risk.

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The first step in estrogen metabolism is the transformation of estradiol to estrone. Estrone is further metabolized by two mutually exclusive pathways, with the main metabolites being 16α-hydroxyestrone (16α-OHE1) and 2-hydroxyestrone (2-OHE1). 16α-OHE1 has been shown to be estrogenic, whereas 2-OHE1 is nonestrogenic (33). These estrogen metabolites are suggested as biomarkers of breast cancer risk. Estrogen metabolism is mediated primarily by cytochrome P450 enzymes, with 2- and 16α-hydroxylation being competitive pathways, so an increase in one metabolite occurs at the expense of the other (1,33). Induction of cytochrome P450 enzymes by lifestyle factors has been proposed to contribute to alterations in estrogen metabolism (1). A greater extent of metabolism via the 2-hydroxylation pathway, and higher 2:16α-OHE1 ratio have been associated with a reduced breast cancer risk in most (18,36), but not all studies (3,32). Two published prospective studies have shown that a higher 2:16α-OHE1 ratio is linked to lower breast cancer risk in premenopausal (23) and postmenopausal women (21).

To date, most observational studies on physical activity and estrogen metabolism have involved young, highly trained athletes (26,27,30). Russell et al. (27) and Snow et al. (30) reported an increase in 2-OHE1 in women who experienced menstrual dysfunction with vigorous aerobic activity. Higher 2-OHE1 was also reported in competitive swimmers compared with recreational runners (25–30 miles·wk^{-1}), whereas both athletic groups had higher 2-OHE1 levels than sedentary controls (26). These studies have methodologic limitations including small sample sizes, inclusion of women with menstrual dysfunction, less reliable measures of estrogen metabolites, and self-report measures of physical activity. Improvements in methodology, including a larger sample of women who have regular menstrual cycles, and objective quantification of chronic physical activity, are needed to further clarify the association between physical activity and estrogen metabolism.

One objective indicator of chronic physical activity is aerobic fitness level. The current gold-standard measure of aerobic fitness is maximal oxygen consumption ($\dot{V}O_{2max}$). Although heredity is believed to account for 25–50% of an individual's $\dot{V}O_{2max}$ level, regular participation in aerobic activity also makes a large contribution and helps to account for the variability seen in the population. Maximal aerobic fitness level is a more objective measure of chronic physical activity than self-reported physical activity pattern. To date, no study has examined the association between aerobic fitness level and urinary estrogen metabolites in normally cycling premenopausal women. The purpose of the present study was to investigate the association between aerobic fitness level and 2-OHE1, 16α-OHE1, and the ratio of 2:16α-OHE1 in premenopausal women. We hypothesized that premenopausal women with high aerobic fitness levels would have a higher nonestrogenic, 2-OHE1 level, and a lower estrogenic, 16α-OHE1 level, a pattern resulting in a higher 2:16α-OHE1 ratio compared with women of average aerobic fitness.

MATERIALS AND METHODS

Participants and Procedures

The study was an extreme group-split design. We recruited participants with distinctly different levels of aerobic fitness to maximize the chance of finding a difference in estrogen metabolites between groups. This study was approved by the research ethics board of the University of Alberta. Participants were recruited from the University of Alberta and its surrounding community by soliciting separately for highly fit and sedentary or recreationally active individuals. The study was restricted to healthy Caucasian women, ages 20–42 yr, who reported regular menstrual cycles (cycle 24–36 d long, and at least 10 cycles in the previous 12 months) and a normal body mass index (BMI) of 18–24. Women were excluded if they had used pharmacologic contraceptives in the past 6 months, had used tobacco products in the past 12 months, were vegetarian, reported an endocrine condition (thyroid or liver disease, or diabetes), reported the use of medication that might interfere with hormonal status (i.e., antidepressants or antibiotics), or had any musculoskeletal conditions that would prevent participation in the aerobic fitness test.

Eligible participants were screened on usual physical activity level using the Godin Leisure Time Exercise Questionnaire (16). Women who reported "strenuous exercise" 3–5 d·wk^{-1} over the past 6 months, and those reporting little or no strenuous or moderate exercise over the past 6 months, were recruited for further testing. Written informed consent was obtained from all eligible participants before participation. Participants completed a Physical Activity Readiness Questionnaire (PAR-Q), then underwent anthropometric measurements and performed a graded exercise test with indirect calorimetry. Maximal oxygen consumption ($\dot{V}O_{2max}$) was used to determine aerobic fitness level. Based on population values for women ages 20–40 yr, cut points for the "high fitness" and "average fitness" groups were ≥48 mL·kg·min^{-1} and ≤40 mL·kg·min^{-1}, respectively (29).

Data Collection

Participant characteristics. Demographics, menstrual history, tobacco use, medication use, and overall health status were collected by questionnaire, using previously validated questions.

Anthropometrics. Participants were weighed in one light layer of clothing and no shoes to the nearest 0.1 kg, and height was measured to the nearest 0.1 cm without shoes. The sum of four skinfolds (triceps, biceps, and subscapular and iliac crest) was measured using calibrated skinfold calipers (Body Care, England) to the nearest 0.2 mm. BMI was calculated using weight (kg) divided by height (m) squared.

Aerobic fitness testing. Eligible participants completed an incremental graded exercise test on a stationary bike (Monark, Sweden). Based on the Godin Leisure Time Exercise Questionnaire, those who reported "strenuous exercise" started at 1.0 kp if body weight was ≥50 kg, or 0.5

kp if body weight was <50 kg, whereas those who reported little or no strenuous or moderate exercise were started at 0.5 kp. Participants were asked to cycle at a self-selected constant cadence, between 60 and 80 rpm, throughout the test. Resistance was increased by 0.5 kp every 2 min until: 1) volitional fatigue, 2) abnormal heart or blood pressure responses, or 3) a drop in cadence >15 rpm. Ventilatory gas exchange was measured throughout the test using indirect calorimetry (Horizon, SensorMedics, Yorba Linda, CA; and True One, Parvo Medics, Sandy, UT). Heart rate was measured continuously using a heart rate monitor (Polar U.S., Woodbury, NY), and recorded at 1-min intervals. Blood pressure was measured before the test and at the 6-min mark. All maximal aerobic fitness tests were performed by a single tester, using a standardized protocol. Criteria for reaching $\dot{V}O_{2max}$ were a plateau in oxygen consumption ($<100\ \text{mL}\cdot\text{min}^{-1}$) during exercise at increasing power output and/or respiratory exchange ratio ≥ 1.1, or volitional fatigue.

Dietary assessment. Usual diet over the past 12 months was assessed using the U.S. NCI Diet History Questionnaire (DHQ) (31) that has been modified for use in a Canadian population. This food frequency questionnaire quantifies the food intake during the past year as well as any dietary supplement use (31). All questionnaires were scanned into TELEform and analyzed using Diet*Cal®, which estimates the average intake over the past year of 26 macro- and micronutrients.

Biologic sampling. Two first-morning urine samples were collected between 0630 and 1030 h after a 10-h water-only fast. Samples were taken between days 4 and 6 and between days 20 and 22 of the same menstrual cycle, with day 1 being the first day of menses. Participants were instructed not to engage in physical activity (beyond activities of daily living) for 24 h before urine sampling. Urine collection was completed at each participant's home, using sterile containers that were brought to the laboratory at the University of Alberta, stored on ice, and processed within 4 h of being received. To prevent the oxidation of metabolites, ascorbic acid ($1\ \text{mg}\cdot\text{mL}^{-1}$) was added to urine before it was aliquoted and stored at −70°C. To determine ovulatory status, saliva was collected daily from day 12 onward of the same menstrual cycle until the start of the next menstrual cycle. Participants chewed on a cotton plug in labeled Salivettes™ (Sarstedt Inc., St-Léonard, Quebec) each morning before brushing their teeth, or before food and drink consumption, and stored the sample in their home freezer until the end of the trial. Samples were then packed in ice, transferred to the University of Alberta, and stored at −70°C. Both written and oral instructions for collection were given to all participants.

Laboratory analysis. 2-OHE1 and 16α-OHE1 were measured using enzyme-linked immunoassay kits (Estramet, Immuna Care Corp. Bethlehem, PA) (19). Because most urinary estrogen metabolites are found in the glucuronide conjugate form, removal of the sugar moiety is required to allow for recognition by the monoclonal antibodies. Samples were incubated for 2 h in deconjugating enzyme and then neutralized. Assay incubation time was 3 h at room temperature. The assay was kinetically read using a Molecular Devices Thermomax plate reader (Molecular Devices, Sunnyvale, CA), and the data were analyzed using Softmax EIA Application software (Molecular Devices). Validity and reproducibility of the EIA kits have been previously demonstrated by comparison with gas chromatography–mass spectrometry (19). All samples, standards, and controls were assayed in triplicate. Samples were initially assayed at a 1:4 dilution because of high estrogen concentration in premenopausal women. Samples were reassayed if they were not within 10% of each other. Samples that were too concentrated or diluted, using standard curve for reference, were reassayed at half concentration or 1:1 or 1:2 dilution, respectively. The 2-OHE1 and 16α-OHE1 urinary levels were standardized by total urinary creatinine. Intraassay coefficient of variation (CV) for 2-OHE1 and 16α-OHE1 was 6.5 and 5.5%, respectively. Interassay CV for the kit and laboratory controls was 9.1% for 16α-OHE1 and 9.7% for 2OHE1. The limit of detection was $0.15\ \text{ng}\cdot\text{mL}^{-1}$ for 2-OHE1, and $0.05\ \text{ng}\cdot\text{mL}^{-1}$ for 16α-OHE1.

Salivary progesterone was measured using a radioimmunoassay (Coat-A-Count, DPC, Los Angeles, CA). Modifications were added to the original protocol to account for the lower level of progesterone found in saliva compared with serum. To improve the sensitivity of the standard curve, provided standards were diluted using the progesterone serum blank to values of 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, and $2.0\ \text{ng}\cdot\text{mL}^{-1}$. All progesterone samples were analyzed in duplicate. The mean of the duplicate measurements was assigned as the sample value. The intraassay and interassay CV was 9.6% and 4.5% for progesterone.

To determine ovulatory status of participants, self-report data of cycle length and serial salivary sampling for progesterone were used. The average of all luteal phase samples was determined and values that fell between 0.11 and 0.2 $\text{ng}\cdot\text{mL}^{-1}$ (12) were used to confirm ovulatory status of that menstrual cycle.

Statistical analysis. Data were analyzed with SPSS version 10.0 software (SPSS Inc., Evanston, IL). Distributions were examined for skewness and outliers, and data were normally distributed. Planned comparisons between the two groups were done using independent *t*-tests. Repeated measures ANOVA, with menstrual cycle phase as the within-subject variable, and fitness level as the between-subject variable, was used to analyze the impact of menstrual cycle phase on estrogen metabolites. Ancillary analysis to explore the relationship between participant characteristics and estrogen metabolites was performed using Pearson correlations. Mean values and standard error are reported.

RESULTS

Flow of participants in the study. A total of 150 women were screened. Of these, 63 were eligible for fitness testing, and 54 completed the fitness test (Fig. 1). Based on test results, 18 women qualified for the high-fitness group, 18 women qualified for the average-fitness group, and 18

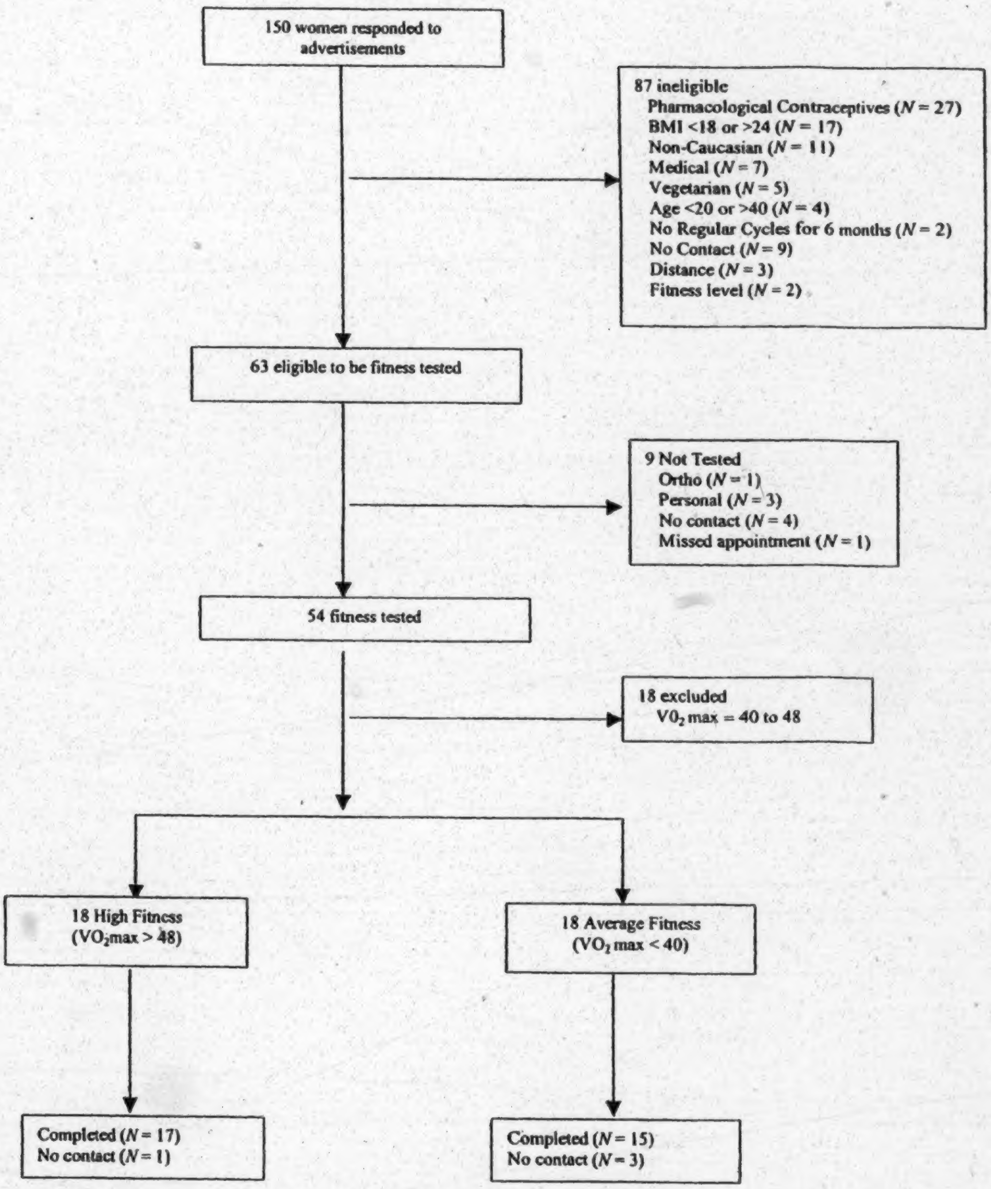

FIGURE 1—Flow chart of study participants.

women were excluded from the study. Overall, 17 women in the high-fitness group and 13 women in the average-fitness group completed the study. Those who did not complete the study failed to provide the requested urine samples.

Baseline characteristics. Table 1 presents the baseline characteristics. The high-fitness group was made up of international-level marathoners and race walkers; national- and provincial-level mountain bikers, road cyclists, and triathletes; and varsity cross-country runners, rowers, and rugby players. During screening with the Godin Leisure Time Exercise Questionnaire (16), the high-fitness group reported an average of 368 min of strenuous physical ac-

TABLE 1. Participant demographics and physical characteristics (mean ± SE).

	High Fitness (N = 17)	Average Fitness (N = 13)	P
Age (yr)	29.5 ± 1.7	27.6 ± 1.6	0.431
Height (cm)	165.2 ± 1.05	166.4 ± 1.72	0.536
Weight (kg)	59.3 ± 1.56	59.9 ± 1.62	0.815
BMI	21.7 ± 0.49	21.7 ± 0.67	0.997
Sum of skinfolds (mm)	36.4 ± 2.69	53.2 ± 3.44	0.001
$\dot{V}O_{2max}$ (mL·kg·min^{-1})	52.8 ± 0.90	35.0 ± 0.93	0.000
Age at menarche	13.2 ± 0.37	12.9 ± 0.38	0.618
Length of cycle	29.3 ± 0.65	27.6 ± 0.80	0.116

tivity per week. The average-fitness group reported an average of 64 min of strenuous physical activity per week, with 9 of 18 (50%) reporting no strenuous physical activity, and 6 of 18 (33%) reporting no strenuous or moderate physical activity on a regular basis. The groups were similar in age, body weight, height, and BMI. As expected, the groups differed in aerobic fitness level, with all participants achieving the criteria for $\dot{V}O_{2max}$. In addition, the average-fitness group had a higher average sum of skinfolds, compared with the high-fitness group. All participants were deemed to be ovulatory using average luteal progesterone levels (i.e., mean of daily samples collected over the luteal phase) and self-report of menstrual cycle.

Planned analysis. Table 2 shows the outcome measures. We found no significant differences between the groups in 2-OHE1, 16α-OHE1, or 2:16α-OHE1 ratio in both the follicular and luteal phase. Nevertheless, the high-fitness group did show a trend towards a higher luteal 2:16α-OHE1 (P = 0.20). There were no significant correlations between aerobic fitness and 2-OHE1, 16α-OHE1, or 2:16α-OHE1 ratio in either the follicular or luteal phases. Again, however, the correlation between aerobic fitness and luteal phase 2:16α-OHE1 ratio approached significance (r = 0.24, P = 0.110).

As expected, menstrual cycle phase had a significant impact on measures of 2-OHE1 ($P < 0.001$) and 16α-OHE1 (P = 0.003), with the concentration of the metabolites being higher in the luteal phase. Further, 2:16α-OHE1 ratio (P = 0.04) was also higher in the luteal phase regardless of fitness level.

The high-fitness group consumed more calories (P = 0.025), carbohydrates (P = 0.009), and dietary fiber (P = 0.014), and had higher levels of total vegetable (P = 0.005), deep yellow vegetable (P = 0.004), and dark green vegetable consumption (P = 0.051). However, none of these dietary factors were associated with 2-OHE1 or 16α-OHE1 levels (data not shown).

TABLE 2. Comparison of hormonal measures for aerobic fitness groups (mean ± SE).

Estrogen Metabolites	High Fitness (N = 17)	Average Fitness (N = 13)	P
Follicular 16α-OHE1	7.55 ± 0.57	7.35 ± 0.81	0.829
Luteal 16α-OHE1	13.11 ± 1.29	14.79 ± 1.63	0.418
Follicular 2-OHE1	9.78 ± 0.88	9.44 ± 0.76	0.782
Luteal 2-OHE1	23.70 ± 2.02	22.11 ± 2.23	0.602
Follicular 2:16α-OHE1	1.37 ± 0.12	1.45 ± 0.20	0.697
Luteal 2:16α-OHE1	1.92 ± 0.13	1.64 ± 0.18	0.203

All metabolite concentrations are expressed as ng·mL^{-1}·mg^{-1} creatinine. The ratio of 2:16α-OHE1 is unitless and is computed by dividing the concentration of 2-OHE1 with the concentration of 16α-OHE1 for the follicular and luteal phases of the cycle.

TABLE 3. Pearson correlations for participant characteristics and hormonal measures.

	$\dot{V}O_{2max}$ (mL·kg·min^{-1})		BMI		Sum of Skinfolds (mm)	
Estrogen Metabolites	r	P	r	P	r	P
Follicular 16α-OHE1	0.024	0.900	0.249	0.177	0.114	0.541
Luteal 16α-OHE1	−0.151	0.417	0.327	0.073	0.386	0.032
Follicular 2-OHE1	0.119	0.524	−0.374	0.038	−0.328	0.072
Luteal 2-OHE1	0.190	0.305	0.057	0.762	0.014	0.941
Follicular 2:16α-OHE1	−0.007	0.970	−0.400	0.026	−0.288	0.116
Luteal 2:16α-OHE1	0.293	0.110	−0.235	0.202	−0.413	0.021

Ancillary analysis. An ancillary analysis was performed to examine the relation between other participant characteristics and hormonal status (Table 3). When levels of estrogen metabolites of all participants were correlated with BMI and sum of four skinfolds, individuals with a higher sum of skinfolds and higher BMI had lower 2-OHE1, lower 2:16α-OHE1 ratio, and higher 16α-OHE1.

DISCUSSION

To our knowledge, this study is the first one to investigate the association between aerobic fitness and 2-OHE1 and 16α-OHE1 in premenopausal women. This study found no statistically significant associations between aerobic fitness and 2-OHE1, 16α-OHE1, or 2:16α-OHE1 ratio. However, there was a trend for the high-fitness group to have a higher luteal 2:16α-OHE1 ratio (P = 0.20), and for high aerobic fitness to correlate with a higher luteal phase 2:16α-OHE1 ratio (r = 0.24, P = 0.110). Moreover, ancillary analyses suggested that sum of skinfolds and BMI were significantly associated with 2-OHE1 and 16α-OHE1 levels.

The key finding from our study was that aerobic fitness was not statistically associated with 2-OHE1 and 16α-OHE1 in premenopausal women. Our results are in contrast to the three previous studies that have investigated the impact of participation in regular physical activity on estrogen metabolism patterns in premenopausal women (26,27,30). Higher levels of 2-OHE1 were seen in competitive swimmers and recreational runners compared with sedentary controls (26), and with "strenuous" training periods in swimmers (27) and varsity rowers (30) who developed menstrual dysfunction.

Several reasons may explain our findings. First, the cut points that were used to compare the two extreme groups may have affected our findings. A threshold effect of aerobic fitness may exist that was not captured by using our cut points. It may be that a significant alteration in estrogen metabolism related to physical activity occurs when moving from low to average aerobic fitness rather than from average to high fitness. Second, alterations in aerobic fitness may not be the pathway through which physical activity may impact estrogen metabolism. Participation in physical activity results in numerous physiological, metabolic, and psychological effects. Regular physical activity may result in alterations in sex steroids and circulating growth factors and binding proteins, as well as changes in body composition, immune function, and antioxidant defenses. These physiological changes may alter breast cancer risk, but not necessarily increase aerobic fitness. In the present study, aerobic fitness ($\dot{V}O_{2max}$) was used as an objective measure of chronic aerobic physical activity. The use an objective measure of current physical activity (i.e., accelerometer) may have added additional important information about the association between physical activity and estrogen metabolites.

The timing of sample collection during the menstrual cycle for analysis of 2-OHE1 and 16α-OHE1 may be an important issue. Our data showed an increase in 2:16α-OHE1 ratio from the follicular to luteal phase in both groups. Some investigators have reported an increase in 2:16α-OHE1 ratio during the luteal phase (4,35), whereas others have not (34). Interestingly, the association between various participant characteristics, such as body weight, BMI, and estrogen metabolites also differed between the follicular and luteal phases. The importance of this observation is unknown. The sampling procedures for urine collection were standardized in this investigation to avoid issues of circadian variation, and seasonal variation, and to standardize the day of menstrual cycle in which samples were collected. Within-person variability for urinary 2:16α-OHE1 is reported to be low with correlations noted between single urine sample and the average ratio of weekly samples across 8 wk ($r = 0.85$, $P < 0.01$) (5). However, controlling for phase of menstrual cycle appears to be an important consideration when examining estrogen metabolites.

In addition, the effects of acute exercise on 2-OHE1 and 16α-OHE1 are not well understood. A series of acute exercise studies by De Cree et al. found no change in 2-OHE$_1$ (7,10), acute increases in 2-OHE$_1$ (8), and acute decreases in 2-OHE$_1$ (9). These data suggest that controlling for the acute effect of physical activity in studies interested in the chronic effects of physical activity may be warranted. In our study, participants were instructed to avoid physical activity for 24 h before urine sample collection. Not only may acute physical activity potentially impact sample collection and analysis, it is possible that physical activity could affect estrogen metabolism in a temporary way that could still accumulate over a lifetime to affect breast cancer risk. Further research into the acute effects of physical activity on estrogen metabolism is warranted.

In an ancillary analysis, we found an association between estrogen metabolites and body composition. This finding is consistent with most (13,23,28), but not all (17), of the previous research. Body composition has also been suggested to be a key determinant of estrogen metabolism in the previous studies on estrogen metabolism related to physical activity, with higher 2-OHE1 levels noted in leaner athletes (26,30). However, no change in the 2:16α-OHE1 ratio was seen with weight loss in a 20-wk lifestyle diet and physical activity intervention in premenopausal women, despite an average body weight loss of 4.5 kg and 400 kcal·wk^{-1} increase in physical activity level, whereas an increase in weight, BMI, and waist-to-hip ratio in the control group had a significant positive correlation with a higher 2:16α-OHE1 ratio (24). A higher concentration of 2-OHE1 or higher 2:16α-OHE1 ratio associated with increased body weight is contrary to previous literature (20) and to our findings. A recent exercise intervention study in postmenopausal women (1) found no association with baseline BMI, body fat, lean body mass, abdominal fat, or subcutaneous fat; a weak positive association between an increase in lean body mass and an increase in 2-OHE1 levels in the exercise group; and a weak inverse relationship between intraabdominal fat and 2-OHE1 in the control group. However, a recent study by Matthews et al. (20) found that although those with a higher BMI (≥25 kg·m^{-2}) and lower levels of self-reported physical activity had a lower 2:16α-OHE1 ratio, women who were active but who also had a higher BMI did not have a reduced 2:16α-OHE1 (20). Overall, physical activity that induces weight loss or body composition change may be more important than physical activity that improves aerobic fitness.

Our study had several strengths. The first strength was the inclusion of women with regular menstrual cycles to avoid the impact of menstrual dysfunction on estrogen metabolism patterns. Participants reported menstrual cycle length and collected daily luteal phase salivary progesterone samples, which were used to discriminate between ovulatory and anovulatory cycles. Although this technique is not absolute due to the variation in progesterone levels within normal cycles, all participants fell within normal values reported for an ovulatory luteal phase (12). Although daily urine collection to determine luteinizing hormone surge and subsequent progesterone levels would have improved the identification of ovulatory cycles, our procedures were less burdensome on participants and more feasible to measure.

A second strength of our study was that we limited recruitment to women who were premenopausal but reproductively mature. Previous studies compared young athletes with older athletes and older controls (26,27). The discrepancy in age, and more specifically reproductive age (i.e., years since menarche), between the two groups may have influenced the findings of comparison in hormones and estrogen metabolism pattern. Only Snow et al. (30) compared rowers and controls of similar age (22–23 yr). Matthews et al. (20) combined pre- and postmenopausal women, whereas Atkinson et al. (1) looked at postmenopausal women.

A third strength of our study was the measurement of aerobic fitness. To get a more objective measure of chronic physical activity level, we determined maximal aerobic capacity ($\dot{V}O_{2max}$) using an incremental cycle ergometer test. Previously, measurement of regular physical activity has been based on self-reported training of individuals or group-training programs, namely swim distance, running mileage, or participation in a varsity rowing program. Self-reported physical activity involves a number of methodological issues, such as overreporting of frequency, duration, and intensity. Metabolic determination of aerobic fitness using a graded exercise test is considered the gold-standard measure of aerobic fitness.

Our study had several limitations. The study had a small sample size, which was further compromised by dropouts. Despite this, the high-fitness group tended to have a higher luteal 2:16α-OHE1 level, a finding consistent with the proposed hypothesis and with previous literature using self-reports of physical activity (2). There is a lack of information on a clinically important difference in 2-OHE1 or 16α-OHE1 levels, as well as limited information on an expected change in these metabolites due to physical activity. With this lack of *a priori* knowledge, it is difficult to determine an appropriate sample size. Our study was powered to detect a difference between the average and highly fit groups of 1.0 standard deviations (a large effect) with an alpha of $P = 0.05$ (two-tailed) and a power of 0.80 based on normative data from Westerlind et al. (34). Our findings suggest that the impact of chronic physical activity on 2:16α-OHE1 level was approximately 0.5 standard deviations (a moderate effect), and future studies might be powered to detect such an effect size until clinically important differences are known.

In addition, our study assessed body composition using skinfold calipers, which is a low-cost, simple method of assessing body composition, although it requires experience on the part of the assessor. Although BMI and sum of skinfolds are both descriptive anthropometric values, BMI as an indicator of subcutaneous fat has limitations, particularly for lean individuals with higher body mass (25).

Although no significant difference in 2-OHE1, 16α-OHE1, or 2:16α-OHE1 ratio was found between women with high versus average aerobic fitness, the trend toward higher 2:16α-OHE1 ratio in the high aerobic fitness group is a promising finding. Future directions for research should focus on women of all levels of aerobic fitness to determine the effect of a wide spectrum of aerobic capacity and, by association, varying levels of physical activity participation on estrogen metabolism patterns. Investigating the impact of body composition and its interaction with physical activity is another important avenue of future research. Furthermore, a prospective intervention study would allow for the quantification of physical activity (i.e., volume and intensity) and the potential effects on estrogen metabolite levels.

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CHAPTER 4 – Study Two

Effects of aerobic exercise training on estrogen metabolism in premenopausal women: A randomized controlled trial.

ABSTRACT

Physical activity has been suggested to alter estrogen metabolism, a proposed biomarker of breast cancer risk, by shifting metabolism to favour production of 2-OHE1. However, to date the majority of studies examining the association between estrogen metabolism and physical activity have been observational. **Purpose:** To examine the effects of a 12-week aerobic exercise training intervention, designed to improve maximal oxygen consumption (VO_{2max}), on 2-OHE1 and 16α-OHE1 in premenopausal women. **Methods:** Following a baseline menstrual cycle, eligible participants (N=32) were randomly assigned to a 12-week aerobic exercise training intervention (N=17) or usual lifestyle control group (N=15) in a 1:1 ratio. Participants were healthy, regularly menstruating, Caucasian women, aged 20-35 years, with a BMI of 18-29.9, not using pharmacological contraceptives, with average or below average fitness ($VO_{2max} < 40$ ml/kg/min). Height, body mass, body composition measured by DEXA and maximal oxygen consumption (VO_{2max}) determined by an incremental cycle ergometer test were measured at baseline and following the intervention. Urine samples were collected in the luteal phase of four consecutive menstrual cycles (baseline plus three intervention menstrual cycles). **Results:** Overall adherence to the training intervention was 91%. The exercise group had a 14 % increase (mean change 4.6 ml/kg/min, CI 3.2 to 6.0) in VO_{2max}, while the control group decreased 3% (mean change -1.0 ml/kg/min, CI -1.9 to 0.0) (difference between groups in mean change 5.6 ml/kg/min, CI 3.7 to 7.2, $p < 0.001$). No significant change in 2-OHE1 (difference between groups in mean change –1.1 ng/ml/mg Cr, CI –

18.1 to 15.8, p=.537), 16α-OHE1 (difference between groups in mean change -0.6 ng/ml/mg Cr, CI –11.1 to 9.9, p=.811) or 2:16α-OHE1 (difference between groups in mean change -0.07, CI –0.69 to 0.54, p=.611) was observed. An increase in lean body mass was associated with a favourable change in 2:16α-OHE1 (r=.43, p=.015). In addition, an inverse association between body fat and 2:16α-OHE1 was noted at baseline (r=-.40, p=.044) and post-intervention (r=.43, p=.015). **Conclusion:** This study examined the effects of an aerobic exercise training intervention on the urinary excretion of estrogen metabolites 2-OHE1, 16α-OHE1 and their ratio, 2:16α-OHE1, in premenopausal women. Engaging in a 12-week aerobic exercise training intervention, which resulted in an increase in aerobic fitness, did not alter estrogen metabolite levels in premenopausal women, however an increase in lean body mass was related to an increase in 2:16α-OHE1. **Keywords:** physical activity, aerobic capacity, fitness, breast cancer, biomarker

INTRODUCTION

Despite the convincing epidemiological association noting physical inactivity as a breast cancer risk factor (1), the underlying biologic mechanisms mediating the association between physical activity and breast cancer risk are not well understood. Cumulative lifetime exposure to sex steroids, particularly estradiol, is thought to play an important role in breast cancer risk (2-5) and physical activity has been suggested to alter levels of sex steroid hormones in both pre- (6, 7) and postmenopausal women (8). Estrogen metabolites, 2-hydroxyestrone (2-OHE1) and 16α- hydroxyestrone (16α-OHE1), have been identified as biomarkers of interest in the research aimed at understanding the mechanisms by which physical activity exerts its protective effects.

The first step in estrogen metabolism is the transformation of estradiol to estrone by oxidation. Estrone is further metabolized to produce two main metabolites, 2-OHE1 and 16α-OHE1, by hydroxylation via competitive pathways, so an increase in one metabolite occurs at the expense of the other (9, 10). Other metabolites have been identified, such as 4-hydroxyestrone and 2-methoxy-estradiol, but these occur in small quantities by comparison and their actions are under investigation (11). 16α-OHE1 has been shown to be estrogenic, while 2-OHE1 is shown to be non-estrogenic (12). The estrogenic effects of 16α-OHE1 were illustrated in a recent study where incubation of human estrogen receptor-positive ovarian cancer cell lines with 16α-OHE1 showed that 16α-OHE1 caused greater proliferative and antiapoptotic effects than estradiol itself (13). Higher 2-OHE1 levels are suggested to have a protective effect, along with a higher 2:16α-OHE1, while higher 16α-OHE1 levels or a lower 2:16α-OHE1 are associated with an increased breast cancer risk (11, 14). In addition to the proposed enhanced cellular proliferation due to stimulation of intracellular estrogen receptors, additional mechanisms for cancer development have also been proposed, such as the production of quinone derivatives of metabolites which may cause DNA changes due to oxidative damage (11).

An association between these estrogen metabolites and breast cancer has been supported by some (10, 15-19) but not all (20, 21) case-control studies, and the evidence from prospective cohort studies suggests a non-significant reduced risk of breast cancer in women with higher levels of 2:16α-OHE1, especially for premenopausal women (14, 22, 23). Physical activity has been suggested to alter estrogen metabolism. Until recently this idea was based on three small studies which pointed to higher 2-OHE1 levels in more active young women, especially those who develop menstrual dysfunction associated with exercise (24-26). Two recent cross-sectional studies suggest that higher self-reported physical activity is associated with an increase in levels of 2-OHE1 and 2:16α-OHE1, however, this association may be related to issues of body composition (27, 28). In addition, Campbell et al. (29) have previously reported a non-significant difference in favour of a higher luteal 2:16α-OHE1 in highly aerobically fit women compared to women with average aerobic fitness, along with an overall inverse association between body composition (i.e. higher BMI and higher sum of skin folds) and 2:16α-OHE1 (29). To date, the effects of an aerobic exercise training intervention on estrogen metabolism in premenopausal women have not been investigated using randomized controlled trial (RCT) methodology. RCTs are considered the strongest research design for determining the causal effects of an intervention on an outcome (30). Therefore, the purpose of the present study was to determine the effects of a 12-week aerobic exercise training program on estrogen metabolites in previously sedentary or recreationally active premenopausal women using RCT methodology. It was hypothesized that the aerobic exercise training program would cause an increase in 2-OHE1 and the 2:16α-OHE. It was further hypothesized that changes in estrogen metabolites would be associated with changes in aerobic fitness and body composition, especially body fat levels.

MATERIALS AND METHODS

Setting and Participant Recruitment

Participants were recruited from the University of Alberta (Edmonton, Alberta, Canada) and surrounding community for a randomized controlled trial of a 12-week aerobic exercise training program compared to usual lifestyle. This study was approved by the Research Ethics Board of the University of Alberta and all participants provided written informed consent prior to participation.

Successful recruitment strategies from the pilot cross-sectional observational study (Study One) were employed and expanded. These included email postings to university campus groups (i.e. Graduate Student Association) and faculties with a high percentage of women (i.e. Nursing and Rehabilitation Sciences), radio interviews and newspaper articles. Word of mouth was also used to recruit participants. The recruitment strategies for all respondents and for those participants who were randomized are presented in Appendix 1.

If initial contact was made via email, the participant was asked to provide a telephone number at which she could be reached and a convenient time to call, at which time the study coordinator administered the Telephone Screening Tool (TST) (Appendix

2). If initial contact was via telephone, the TST was administered by the coordinator, if available. Otherwise, relevant contact information and the best time of day to call were recorded on the study telephone log. The study director then made contact with the potential participant as soon as possible and administered the TST. Once a participant was deemed eligible, a detailed description of the study was provided. If the participant was interested in continuing with the screening process, a maximal aerobic fitness test was scheduled

Eligibility criteria

The eligibility criteria for the trial were: 1) female, 2) Caucasian, 3) sedentary or recreationally active (not engaging in vigorous-intensity exercise for 20 minutes or more, three or more times per week in the past six months with no history of significant aerobic training in the past year) and "average" aerobic fitness, determined as maximal oxygen consumption (VO_{2max}) < 40 ml/kg/min, 4) 20 to 35 years of age, 5) self-reported regular menstrual cycles (cycle 24-36 days long, and at least 10 cycles in the previous 12 months), 6) normal or overweight body mass index (BMI) of 18-29.9, 7) no use of pharmacologic contraceptives (past six months), 8) no use of tobacco products (past 12 months), 9) not vegetarian, 10) no self-reported endocrine condition (thyroid or liver disease, or diabetes), 11) no use of medication that might interfere with hormonal status (i.e. anti-depressants or antibiotics), 12) free of musculoskeletal conditions that would prevent participation in an aerobic exercise program, and 13) geographically accessible, 14) Not planning to be away for more than 7 consecutive days over the 12 week intervention, 15) willing to be randomized. The rationale for the eligibility criteria is provided in Appendix 3.

Study Protocol

Participants met with the study coordinator to go over the details of the study (Figure 1). Consent (Appendix 4), Participant's Responsibilities (Appendix 5), and General Demographic (Appendix 6) forms were completed, prior to beginning the aerobic fitness test. These forms provided self-reported information on demographics, menstrual history, medication use, and overall health status. Participants completed a Physical Activity Readiness Questionnaire (PAR-Q) (31) and underwent anthropometic measurement, prior to performing a graded exercise test to determine aerobic fitness level. The participants incurred no costs as a result of the study. All testing costs were covered by the study, along with parking costs for testing and exercise sessions. Participants were not monetarily compensated beyond these provisions.

Anthropometrics. Participants' body mass and height were measured in light clothing without shoes to the nearest 0.1 kg and 0.5 cm, respectively. Waist and hip circumference were measured to the nearest 0.1 cm with an inelastic tape at the narrowest part of the torso and the maximal part of the buttocks, respectively. These measures were also completed at 6-7 weeks (mid-point) and at 12 weeks (end of study) from randomization. At a separate visit, body composition was assessed by dual energy x-ray absorptiometry (DEXA) to determine percent body fat, fat mass in kg, and lean body mass in kg. For the DEXA, mandatory quality control procedures and body composition calibration were completed according to the manufacturer's instructions and all scans were performed by the same trained technician. The DEXA was also repeated at the end of the study by the same technician. Fat distribution was examined in the abdominal region, from the upper border of L2 to the lower border of L4 (android), and in the hip region between the superior iliac spines and sciatic tubers (gynoid) (32).

Aerobic Fitness. Aerobic fitness was determined by a maximal oxygen consumption (VO_{2max}) test using an incremental graded exercise on a stationary bike (Monark, Sweden). Participants were asked to cycle at a self-selected constant cadence, between 60-80 revolutions per minute (rpm), and maintain this throughout the test. Resistance was increased by 30 watts (W) every two minutes until the participant achieved a respiratory exchange ratio (RER) of 1.0, and then increased by 30 W every minute until: 1) volitional fatigue or 2) a drop in cadence with an increase in resistance (> 10 rpm for 30 seconds). Ventilatory gas exchange was measured throughout the test using indirect calorimetry using a calibrated metabolic measurement system (True One, Parvo Medics, Sandy, Utah), calibrated using know gas concentrations and flowmeter calibration for volume. Heart rate was measured continuously using a heart rate monitor (Polar USA, Woodbury, New York), and recorded at one minute intervals. Blood pressure was measured on the upper arm using a manual spygmometer and stethoscope, prior to the test and at the approximated mid-point of the test (i.e. the six minute mark). All maximal aerobic fitness tests were performed by the same tester using a standardized protocol. Criteria for reaching VO_{2max} were: a plateau in oxygen consumption (<100 ml/min) during exercise at increasing power output and/or respiratory exchange ratio ≥ 1.1. "Average" aerobic fitness level based on population values for women ages 20–40 yr, was set at $VO_{2max} \leq 40$ ml/kg/min (33). Those who scored above this value were thanked for their participation but not enrolled in the study. All participants completed another exercise test at the end of the study, while those randomized to the exercise intervention also completed an additional test at mid-point to optimize the exercise prescription.

Dietary Assessment. Usual diet was assessed using a three-day diet record, completed on two weekdays and one weekend day in the follicular phase of the first and fourth menstrual cycle. Data were entered by a Registered Dietician into a database that has been modified for use in a Canadian population (Food Processor II Nutrient Analysis Program TM, Esha Research, Salem, OR). Energy and macronutrient (e.g. protein, fat, carbohydrate) intake, along with selected dietary factors that have been associated with estrogen metabolism (i.e. *Brassica* vegetables, soy, and fiber intake) were determined.

Exercise Assessment. Usual physical activity was assessed using the Godin Leisure Time Exercise Questionnaire (34). At the time of screening participants were asked about bouts of "strenuous", "moderate" and "mild" physical activity in the past seven days. This was repeated in the last week of the intervention.

Randomization. Following completion of baseline measurements participants were randomly assigned to either the "exercise" (12- week aerobic activity program) or "control" (usual lifestyle) group using a computer generated random numbers list (Statmate, Version 1.01, 1998) in a 1:1 ratio. A permuted block design was used to generate the allocation sequence. The block sizes were random and determined by a trained research assistant that had no involvement in the study. Randomization was stratified on BMI (< 25 or ≥25). A trained research assistant generated the group assignments in sequentially numbered and sealed opaque envelopes. Once all baseline measurements were completed, the envelopes were opened in sequence by the trial coordinator. The participant number and allocation were recorded on the envelope prior to filing the opened envelope. The participant was then informed of the allocation by the research coordinator.

Intervention Group. Those randomized to the intervention arm began the exercise program in the early follicular phase of the next menstrual cycle (day 1-5). The aerobic exercise training intervention was a 12-week individualized, progressive, moderate-to-vigorous intensity, supervised, aerobic training program aimed at improving aerobic fitness, measured as improvement in VO_{2max}. The greatest improvement in VO_{2max} occurs with high intensity training corresponding to maximal intensities (35). An effective method of performing high intensity training is through the use of intervals, which allows participants to accumulate longer durations at higher intensities (36). Due to the fact that participants were sedentary and some were naïve to aerobic training, the program began with moderate intensity training in order to build an aerobic base and introduce participants to the training program. This was followed by the addition of vigorous intensity intervals after four weeks. The first type of interval (Interval 1) was designed to introduce participants to more vigorous intensity exercise as a progression toward to maximal intensity intervals. The second type of interval (Interval 2) was designed to have participants working at a maximal intensity for the longest accumulated duration possible. Overall, the program was designed to minimize the risk of injury, maximize participant compliance, and achieve the greatest increase in VO_{2max} possible (35, 36).

Individualized exercise programs for each participant were determined from the baseline and mid-point aerobic fitness tests (Appendix 7). Intensity of the exercise bouts was based on power output (watts) at ventilatory equivalents for oxygen (V_E/VO_2) and carbon dioxide (V_E/VCO_2). This method of exercise prescription uses metabolic parameters as the basis for training intensity, rather than percentage of maximal oxygen consumption (VO_2max) or maximum heart rate, to minimize training at different relative intensities between participants (37). V_E/VO_2 is defined as the lowest point in the ratio of ventilation (V_E) and oxygen consumption (VO_2) prior to a systematic increase without a concurrent rise in the ventilatory equivalent for oxygen, while V_E/VCO_2 is defined as the lowest point in the ratio of ventilation (V_E) and carbon dioxide production (VCO_2) prior to a systematic increase (38). Exercise participants completed an additional VO_{2max} test at mid-point (Week 7) to account for possible improvements in aerobic fitness elicited by the program and to ensure optimal exercise prescription throughout the entire intervention.

Specifically, the intervention was divided into three distinct blocks:

Week 1-4: Participants performed three sessions per week of base aerobic training progressing from 20-40 minutes on a stationary bike (Lifestyle Fitness, 9500HR; Life Fitness, Franklin Park, IL). Intensity was based on wattage corresponding to approximately 25% higher than V_E/VO_2. All sessions included an additional warm up (5-10 minutes) and cool down (5 minutes) on the equipment used.

Week 5-8: Participants performed four sessions per week. Two sessions were base aerobic training sessions for 30-45 minutes as described previously. At least one of these sessions had to be performed on a stationary bike, and the other could be on other aerobic equipment (treadmill or elliptical). Two additional interval sessions were completed on a stationary bike. One interval session (Interval 1) consisted of two 10-minute intervals at a power output corresponding to V_E/VCO_2, with 10-minutes of easy cycling between the intervals. The other interval session (Interval 2) consisted of intervals at a power output corresponding to VO_{2max} for 30 seconds followed by 30 seconds of easy pedaling, building from two sets of 10 intervals to one set of 20 intervals. All sessions included an additional warm up (5-10 minutes) and cool down (5 minutes) on the equipment used. Participants were also encouraged to stretch following a training bout.

Week 9-12: Participants performed four sessions per week, with two base aerobic training sessions for 30-45 minutes and two intervals sessions similar to Week 5-8. Exercise prescription was updated based on the mid-point VO_{2max} test. During one base aerobic training session per week, performed on a stationary bike, participants were asked to add high resistance cycling on the ergometer by self-selecting a decreased pedaling cadence and increased resistance to subjectively simulate climbing a hill. Participants rode a usual base training power output for eight minutes, then switched to high resistance for two minutes, and repeated this four times (40 minutes session). In addition, Interval 2 was changed to two minutes at a power output corresponding to VO_{2max} and three minutes of easy pedaling, four times. All sessions included an additional warm up (5-10 minutes) and cool down (5 minutes) on the equipment used. Participants were also encouraged to stretch following a training bout.

The intervention included a strong behavioural support component to achieve high adherence. All sessions were supervised by an exercise trainer (i.e. degree in Physical Education or Kinesiology, CPR training, and related experience) who was trained in administering exercise protocols, monitoring normal and abnormal responses to exercise, participant motivation, clinical trial methods, and emergency management. Sessions were completed at the Behavioural Medicine Fitness Centre (BMFC) in the Research

Transition Facility at the University of Alberta. Attendance at each session was recorded by an exercise trainer and a missed session was followed up with a telephone call or email (based on identified participant preference) to determine the reason for the missed session. Attempts were made to re-schedule the missed session to ensure the prescribed number of sessions was achieved for that week. Individualized feedback was provided on a weekly basis by the research coordinator after reviewing each exercise file to monitor the adherence to the exercise prescription and to ensure it was challenging but achievable for the participants. In addition, from week 5-12, the research coordinator supervised one session per week of interval training (Interval 2), which corresponded to the hardest intervals. Also, the centre was open 7 am until 7 pm Monday – Friday and 9am until 1 pm on Saturdays and holidays to allow participants to exercise at a time that was convenient. Participants booked each session to ensure equipment would be available, and average heart rate and rating of perceived exertion were recorded for all sessions

Control Group. Participants in the control group were asked to maintain their usual activity levels for the duration of the study. Following the control cycle, the first day of the next menstrual cycle was used as the reference start date for participants in the control group. Upon completion of the 12-week post-intervention measurement, participants from the control group were offered access to the Behavioural Medicine Fitness Centre for four weeks and given guidance for starting an individualized exercise program.

Biological Sampling

Sampling occurred over four consecutive menstrual cycles, with cycle one serving as the baseline cycle, and cycle four serving as the post-intervention cycle.

Urine samples. Urine samples were collected for analysis of estrogen metabolites, 2-OHE1 and 16α-OHE1. Over each of the four menstrual cycles observed during the research study, two first morning urine samples were collected between 0630 and 1100 following a 10-hour water-only fast. Samples were taken between Day 4-6 and between Day 20-22 of the menstrual cycle, with Day 1 being the first day of menses. Participants were instructed not to engage in physical activity (beyond activities of daily living) for 24 hours prior to urine sampling. Urine collection was completed at the participants' home using sterile containers and brought to the laboratory at the University of Alberta, where it was stored at 4-8 °C and processed within four hours of being received. To prevent the oxidation of metabolites, ascorbic acid (1mg per ml) was added to urine prior to being aliquoted and stored at -70°C.

Saliva Samples. First morning fasted saliva samples were collected during the mid-luteal phase of each of the four menstrual cycles to allow for determination of average mid-luteal progesterone levels. Participants were instructed to passively drool into a provided tube every other day for six days specified by the investigators. This was determined on an individual basis for each participant by using the previous menstrual cycle length as a basis for the length of the subsequent cycle and subtracting 14 days from the projected end day of that cycle to determine day of ovulation. The mid-luteal phase samples were assigned to day five, seven and nine of the projected 14 day luteal phase. All samples were stored in a home freezer prior to transport on ice to the University of Alberta, where all samples were stored at -70°C.

Laboratory Analysis. 2-OHE1 and 16α-OHE1 were measured using enzyme-linked immunoassay kits (Estramet, Immuna Care Corp. Bethlehem, PA) (39). Since estrogen metabolism levels are higher in the luteal phase (40-42), and based on previous results which show a non-significant difference in favour of higher luteal 2:16α-OHE1 in women with high levels of aerobic fitness (29), the luteal phase urine sample was used for analysis of estrogen metabolites. Since most urinary estrogen metabolites are found in the glucuronide conjugate form, removal of the sugar moiety is required to allow for recognition by the monoclonal antibodies. Samples were incubated for two hours in deconjugating enzyme, and then neutralized. Assay incubation time was three hours at room temperature. The assay was kinetically read using a Molecular Devices Spectra Thermo max microplate reader (Molecular Devices, Sunnyvale, CA) and the data were analyzed using Softmax Application software (Version 2.35 Molecular Devices, Sunnyvale, CA). Validity and reproducibility of the enzyme-linked immunoassay kits have been previously demonstrated by comparison to gas chromatography-mass spectrometry (39). All samples, standards, and controls were assayed in triplicate. Samples were initially assayed at a 1:4 dilution because of high estradiol concentration in premenopausal women. Samples were re-assayed if the coefficient of variation (CV) was greater than 10%. Re-assay was required for 19 samples. Samples that were too concentrated or dilute, using standard curve for reference, were re-assayed at 1:8 or 1:2 dilution, respectively. The 2-OHE1 and 16α-OHE1 urinary levels were standardized to total urinary creatinine (ng/ml/mg Cr). Creatinine was measured by colorimetric Microplate assay (Oxford Biomedical Research, Oxford, MA). Intra-assay coefficient of variation (CV) for 2-OHE1, 16α-OHE1 and creatinine were 4.3%, 4.9% and 3.7%, respectively. Inter-assay CV for the kit controls was 5.8% for 2-OHE1 and 4.9% for 16α-OHE1. The limit of detection was 0.15 ng/ml for 2-OHE1 and 0.05 ng/ml for 16α-

OHE1. Total estrogen metabolite concentration (EMC) is the combined amounts of 2-OHE1 and 16α-OHE1.

Salivary progesterone was measured using a competitive enzyme immunoassay (Salimetrics, State College, PA). All progesterone samples were analyzed in duplicate. The mean of the duplicate measurements was assigned as the sample value. The intra-assay and inter-assay CV for kit controls was 7.8 % and 6.2 %, respectively. In order to determine ovulatory status of participants, self-report data of cycle length and mid-luteal salivary sampling for progesterone were used The average of all luteal phase samples per cycle was determined and values that fell between 0.11-0.2 ng/ml (43) were used to confirm ovulatory status of that menstrual cycle. The limit for detection was 0.005 ng/ml

Sample Size Calculation and Statistical Analysis

A clinically important difference in estrogen metabolites has not been identified and there is little reliable literature which outlines the expected achievable changes in estrogen metabolism with exercise training. Based on an increase in 2:16α-OHE1 in a dietary intervention trial in premenopausal women (44) and taking into account a possible 10% dropout rate, it was estimated that 16 participants per group were needed to detect a 16% difference in 2:16α-OHE1 using an independent t-test with a power of .80 and a two-tailed alpha of 0.05.

Data were analyzed with SPSS version 12.0 software (SPSS Inc., Evanston, Illinois). Distributions were examined for skewness and outliers. The assumption of normality was not met for one 2-OHE1 value (cycle one, skewness = 2.6) and all measures of 16α-OHE1 (cycle one to four, skewness = 3.5, 1.2, 2.4 and 2.0, respectively). Therefore, both untransformed and transformed data were analyzed. For transformed data, planned comparisons between the two groups were analyzed for the primary time point of interest (cycle one to cycle four) using independent t-tests, and secondary exploratory analysis for change over time using repeated measures ANOVA. For the repeated measures ANOVA, the between subjects factor was group assignment (i.e., exercise versus control) and the within subjects factor was time (i.e., the four cycles). For untransformed data, planned comparisons between the two groups were analyzed using repeated measures ANOVA and the Mann-Whitney tests. For independent t-tests and Mann-Whitney tests, change scores (i.e. post-intervention value minus initial value) were computed by subtracting Cycle 4 from Cycle 1.

Associations between aerobic fitness/body composition and estrogen metabolites were analyzed by Spearman Rank order (untransformed data) and Pearson correlations (log transformed data) at baseline, post-intervention, and for change scores. Additional partial correlations were performed to control for potential confounders. Independent t-tests were used for exploratory subgroup analysis to examine variations in estrogen metabolites in response to exercise training based on baseline and change scores for aerobic fitness, body composition, and menstrual dysfunction using untransformed 2:16α-OHE1. The between subjects factors were group assignment (i.e. exercise versus control) and the factor of interest (e.g. initial BMI). A Group X Factor interaction would indicate a differential effect of group assignment based on the factor of interest. Mean value and standard error are reported for continuous variables, and number and percentage are reported for categorical variables. Last-observation-carried–forward was used to address missing data.

RESULTS

Flow of Participants through Trial

Flow of the participants through the trial is presented in Figure 2. Of 192 women screened for the trial, 52 were eligible for fitness testing and 46 completed the fitness test. Based on fitness test results, 34 women were eligible, and 32 completed all baseline measures and were willing to be randomized. Overall, 17 women were randomized to the exercise intervention and 15 women were randomized to the control group.

All participants provided baseline samples. For cycle 2, 30 participants provided samples. For cycle 3, 31 participants provided samples. For cycle 4, 29 participants provided samples. One participant provided only baseline and post-intervention samples, therefore her baseline values were carried forward for cycle 2 and cycle 3. One participant did not provide a sample for cycle 2, and analysis of the cycle 3 and 4 samples did not provide valid results, therefore the baseline values were carried forward for all time points. For the three participants that did not provide the final sample, the cycle 3 value was carried forward for the statistical analysis of this data. All women returned to complete the end of study testing.

Baseline Characteristics of Participants

Table 1 presents the baseline participant characteristics. The groups were balanced on all demographic, anthropometric, aerobic fitness, and reproductive measures. Participants were on average, 25.9 years of age with a body mass of 65.1 kg. The average BMI for participants was 23.1 kg/m^2, with an average waist-hip-ratio (WHR) of 0.74 and percent body fat of 33.2 %. At baseline, participants reported an average of 57.9 minutes of moderate and 44.5 minutes of strenuous physical activity per week.

Seven participants (22%) met public health guidelines for physical activity (i.e. 30 minutes of moderate physical activity at least five days of the week) (31). Overall, participants had a VO_{2max} of 32.7 ml/kg/min or 2.12 L/min, indicating below average fitness for their age group (33). All participants achieved the criteria for VO_{2max} and the main reason for stopping the test was volitional fatigue. Only one VO_{2max} score had to be adjusted due calibration error. No participant had a first-degree relative with breast cancer.

Baseline Estrogen Metabolites and Preliminary Associations

At baseline, no difference in 2-OHE1, 16α-OHE1, 2:16α-OHE1 or total EMC was seen between the exercise and control group (Table 3). Baseline 2:16α-OHE1 was associated with percent body fat (r=-.40, p=.044), hip region percent fat (r=-.39, p=.028) and abdominal region percent fat (r=-.36, p=.044), while an association with WHR approached significance (r=-.34, p=.055) (Table 3). There was no association between baseline body composition, physical activity, aerobic fitness and 2-OHE1. A significant positive association was found between 16:OHE1 and abdominal body fat (r=.38, p=.031 for percent body fat; r=.43, p=.013 for total body fat), while a positive association between overall body fat and 16:OHE1 approached significance (r=.33, p=.067 for percent body fat). At baseline, one participant had high values of both 2-OHE1 and 16:OHE1 (137.9 ng/ml/mg Cr and 77.1 ng/ml/mg Cr, respectively), however, excluding her from the data did not substantially change the results.

Adherence to the Intervention

Participants attended, on average, 40 of 44 prescribed exercise sessions for an overall adherence rate of 91%, ranging from 64-100% (Table 4). Adherence was: 96%

for Week 1-4, 93% for Week 5-8 and 81% for Week 9-12. Fourteen participants (82%) completed greater than or equal to 80% of the prescribed exercise sessions, which was set as an adherence cutoff for successful delivery of the exercise intervention. Based on the Borg scale of rating of perceived exertion (45), participants reported the intensity for the base aerobic sessions as "somewhat hard", the Interval 1 session as "hard" and the Interval 2 sessions as "very hard"/"very very hard" (Table 5). At the end of the intervention, the exercise group reported an average of 155.0 minutes of strenuous and 79.7 minutes of moderate physical activity per week on the Godin Leisure Time Exercise Questionnaire, which was a 377% increase in strenuous and 18% increase in moderate minutes from baseline. All sessions completed as part of the intervention were recorded as "strenuous" activity, accounting for 140 minutes of strenuous activity during weeks 4-12 of the intervention (i.e. not including warm up and cool down, two base training sessions lasting 40 minutes each and two intervals sessions lasting 30 minutes each) . Two participants reported two additional strenuous activity bouts per week (40 to 60 minutes) and two participants reported one additional strenuous activity bout per week (40 to 45 minutes). The control group reported an average of 37.3 minutes of strenuous and 47.3 minutes of moderate physical activity per week at the end of the study, which represents a decrease of 35% and 7% from baseline, respectively.

Adverse Events

Three participants in the exercise group (18%) experienced an adverse event compared to one adverse event in the control group (7%). The adverse events in the exercise group were influenza (n=1), broken wrist (n=1), and car accident (n=1), none of which were deemed to be associated with the intervention. The adverse event in the control group was mononucleosis (n=1).

Effects of Aerobic Exercise Training on Estrogen Metabolism

The AET intervention had no effect on levels of 2-OHE1 and 16α-OHE1 or 2:16α-OHE1 (Table 2). No difference in 2-OHE1, 16α-OHE1 or 2:16α-OHE1 (Figure 3) was seen between groups over the course of the intervention demonstrated by a non-significant group by time interaction for untransformed 2-OHE1 (F = .03, p=.860), 16α-OHE1 (F = .00, p=.962), 2:16α-OHE1 (F= .54, p=.466) and total EMC (F=.01, p=.930) or log transformed 2-OHE1 (F = .24, p=.627), 16α-OHE1 (F= .07 p=.797), 2:16α-OHE1 (F = .00, p=.987) and total EMC (F=.00, p=.930). Differences between groups in changes from baseline to post-intervention were also not observed for 2-OHE1 (mean change -1.1, CI –18.1 to 15.8, p=.835), 16α-OHE1 (mean change -0.6, CI –11.1 to 9.9, p=.806), 2:16α-OHE1 (mean change -0.07, CI -0.69 to 0.54, p=.925) or total EMC (mean change -1.7, CI –27.7 to 24.4, p=.925).

Effects of Aerobic Exercise Training on Aerobic Fitness

The effects of the AET intervention on aerobic fitness are shown in Table 6. The exercise group increased VO_{2max} by 4.6 ml/kg/min or 0.27 L/min, while the control group decreased by 1.0 ml/kg/min or 0.06 L/min (mean group change = 5.6 ml/kg/min [3.7 to 7.2], p <.001) (Figure 4). This represents a 14% increase in VO_{2max} for the exercise group and a 3% decrease for the control group. An increase in maximum power output was seen in the exercise group compared to the control group (p <.001). This represents a 20% increase in maximum power output in the exercise group. Increases in oxygen consumption at V_E/VO_2 (p=.001) and V_E/VCO_2 (p=.001), and power output at V_E/VO_2 (p

<.001) and V_E/VCO_2 (p=.001) were also seen in the intervention group compared to the control group, indicating an overall improvement in aerobic exercise capacity.

Effects of Aerobic Exercise Training on Body Mass and Body Composition

The AET had no significant effect on body composition as measured by body mass (Figure 5), BMI, waist and hip circumference or WHR (Table 7). However, compared to the control group, the exercise intervention group lost fat mass (-1.2 kg, CI –2.2 to –0.2) and gained lean mass (0.9 kg, CI 0.2 to 1.6). In terms of fat distribution, the exercise group lost fat in both the hip (-0.3 kg, CI –0.5 to –0.1) and abdominal regions (-0.1 kg, CI –0.2 to 0.0). No significant change in energy intake or macronutrients was seen in either group across the course of the intervention (Table 8). There was no difference between groups for the number of participants who reported *Brassica* vegetable intake at baseline (exercise 44% and control 50%, p=.732) and post-intervention (exercise 25 % and control 50%, p=.193) or soy intake at baseline (exercise 13% and control 8%, p=.626) and post-intervention (exercise 8% and control 8%, p=.844).

Associations Between Changes in Estrogen Metabolites and Changes in Aerobic Fitness/Body Composition

Improved VO_{2max} was not associated with changes in estrogen metabolites levels (Table 9). However, a positive association between increases in total lean body mass and 2:16α-OHE1 was found (r=.43, p=.015). When controlling for change in body fat percentage, this association remained significant (r=.36, p=.046).

Associations Between Post-intervention Estrogen Metabolites and Post-intervention Fitness/Body Composition

Post-intervention 2:16α-OHE1 had an inverse association with BMI (r=-.39, p=.028), body fat (r=-.40, p=.027 for percent body fat; r=-.39, p=.028 for total body fat), and hip region fat (r=-.36, p=.044 for percent fat ;r=-.36, p=.045 for total fat) and abdominal region fat (r=-.38, p=.034 for percent fat; r=-.35, p=.047 for total fat) (Table 10). No associations were observed with post-intervention levels of 2-OHE1 and 16α-OHE1.

Associations Between Estrogen Metabolites and Fitness/Body Composition for the Exercise Group Alone

Results from analysis of the exercise group alone are shown in Table 11. Changes in aerobic fitness and change in 2-OHE1,16α-OHE1 or 2:16α-OHE1 were related. A positive association between gain of lean mass and change in 2:16α-OHE1 level (r=.53, p=.029) was found. In addition, a change in hip region fat was associated with a change in both 2-OHE1 (r=-.49, p=.047) and 16α-OHE1 (r=-.48, p=.050).

Ovulatory Status

Using Day one as the onset of menses, samples were collected on Day 21, 23 and 24 of cycle one and Day 21, 23 and 25 of cycle four. The majority of participants maintained consistent menstrual cycle length within the normal range (24-36 days) (46) and had mid-luteal progesterone values that were between 0.11-0.2 ng/ml which suggest a positive ovulatory status for that menstrual cycle (43). Menstrual cycle length was recorded for all participants across four cycles, while mid-luteal salivary progesterone samples were collected in cycle 1 and cycle 4 (Table 12). Mid-luteal progesterone samples were not available for three participants in cycle 1 (two in control group and one in exercise group) and 12 participants in cycle 4 (6 in control group and 6 in exercise group). Of the 32 menstrual cycles observed in cycle 1, all cycles fell within the normal cycle length, and four cycles were below the set average progesterone values (two in exercise group and two in control group). For cycle 2, of the 32 cycles observed, two cycles were shorter than 24 days (both in exercise group) and one cycle was longer than 36 days (in control group). For cycle 3, all cycles were at least 24 days long and four cycles were longer than 36 days (three in exercise group and one in control group). For cycle 4, all cycles were at least 24 days long and one cycle was longer than 36 days (in exercise group), and two cycles were below the set average progesterone values (one in exercise group and one in control group). Missing samples were either not provided by the participant, noted to be contaminated by participant (i.e. participant identified that this sample was taken after eating or brushing teeth), or did not yield sufficient amounts of saliva to allow for analysis. Overall, possible menstrual dysfunction (i.e. anovulation or LPD) at some point during the intervention is suggested in 8 participants overall, split between six in the exercise group and two in the control group.

Ancillary Subgroup Analysis

Specific baseline characteristics and changes in characteristics previously associated with estrogen metabolism were examined as effect modifiers for hypothesis generation (Table 13). No significant interaction between group and selected participant characteristics were found for change in 2:16α-OHE1.

DISCUSSION:

To date, there are no published randomized controlled trials examining the effects of an aerobic exercise training intervention on the urinary excretion of estrogen metabolites 2-OHE1 and 16α-OHE1 or their ratio, 2:16α-OHE1, in premenopausal women. The aerobic exercise training program caused a significant improvement in aerobic fitness and lowered body fat levels, but there were no significant effects on 2-OHE1, 16α-OHE1, or 2:16α-OHE1. This data does not support the hypothesis that an improvement in aerobic exercise capacity favourably alters 2:16α-OHE1.

The primary finding of the present study is that 12 weeks of aerobic exercise training, sufficient to induce an improvement in maximal oxygen consumption, had no significant effects of estrogen metabolism. This finding is consistent with the only two previous studies that have examined the effects of a physical activity intervention on levels of 2-OHE1, 16α-OHE1, or their ratio, 2:16α-OHE1 (47, 48), but is inconsistent with several observational studies that suggest physical activity is associated with estrogen metabolite levels (24-27). Atkinson et al. (47) found no difference in estrogen metabolite levels following a randomized controlled trial of a 12-month moderate intensity (60-70% of maximal heart rate for 45 minutes) aerobic intervention in 170 postmenopausal women, in which the exercise group had a 12% improvement in VO_2max (49). Pasagian-Macaulay et al. (48) also found no difference in 2:16α-OHE1 between the intervention and control groups during a 20-week group-based weight loss lifestyle intervention in older premenopausal women (age 44-55), in which the intervention group lost body mass, reduced dietary fat intake, and increased self-reported physical activity level by 400 kcal/week (mainly through walking). Together, the results from this study and the other two intervention studies (47, 48) suggest that exercise and physical activity interventions may not have any significant impact on estrogen metabolism in women.

Therefore, the associations reported in observational studies may be spurious. The observational studies have suffered from a number of methodological limitations. In the three early studies (24-26), sample sizes were small (i.e. five to seven participants per group), menstrual function was not standardized (resulting in comparisons between individuals with and without menstrual dysfunction, and women with different reproductive ages), self-reported physical activity measures were used (i.e. swim distance, running mileage, participation in a varsity-rowing program) and the studies used an older version of radioimmunoassay (24, 25) or administration of a labeled tracer (26) to measure estrogen metabolites, which are viewed to be not as valid as the newer solid-phase enzyme immunoassay (39, 48, 50, 51). Methodological improvements have been applied to three more recent studies (27-29), namely the use of larger sample sizes, standardized menstrual status and analysis of estrogen metabolites using a newer solid-phase enzyme immunoassay. However, all have used BMI as a measure of adiposity, which is problematic, especially for more athletic populations (52). Two studies (27-29) used self reports of physical activity levels, which suffer from a number of methodological issues, in particular over reporting of frequency, duration and intensity (53). One study (29) used an objective measure of chronic exercise, maximal oxygen consumption (VO_{2max}).

An association between estrogen metabolites and baseline body composition was observed, which is consistent with some (25, 26, 28, 54-56), but not all studies (57). At baseline, inverse associations between 2:16α-OHE1 and percent body fat were noted. This finding is consistent with the lower 2-OHE1 levels reported in obese individuals compared to those of normal body mass (56), and in women with a higher BMI and higher body fat, regardless of aerobic fitness level (29). In addition, higher 2-hydroxylation was seen in athletes who were leaner (25, 26) and in anorexic women compared to normal body mass and obese women (55). However, Jernstrom et al. (57) found no association between 2-OHE1, 16α-OHE1 or 2:16α-OHE1 and height, body mass or BMI in a cross-sectional study of premenopausal women.

An interaction between body composition and physical activity in relation to estrogen metabolites has also been suggested. Matthews et al. (28) found that in women who reported low levels of physical activity, those with a higher BMI (> 25.0) had lower 2:16α-OHE1, than those with a lower BMI (<25.0). However, women with a higher BMI (>25.0) who reported being physically active maintained a higher 2:16α-OHE1, consistent with women with a lower BMI (<25.0) who also reported being physically active. A similar finding by Bentz et al. (27), which showed a positive association between MET-hours per day of physical activity, 2-OHE1 levels and 2:16α-OHE1, independent of BMI, suggests that overweight women may also benefit more from physical activity in terms of positive alterations in estrogen metabolism. However, when women were split into overweight (BMI >25) and normal weight (BMI < 25), the association between MET-hours per day and estrogen metabolites remained only for the overweight group. The authors point out the issues surrounding the measurement of body composition using BMI and that a number of those in the higher BMI group were athletic and likely did not have levels of adiposity which may account for their findings (27).

Further elucidation of the association between physical activity, body composition and estrogen metabolites would be achieved by prospective measurement of body composition changes and improved methods of assessing body composition. While body mass did not change in either group over the course of the present intervention, the exercise group had a significant increase in lean body mass, with a decrease in total fat mass and a decrease percent body fat. A mean body mass loss of 1.29 kg over 12-months in the intervention by Atkinson et al. (47) and 4.5 kg over 20-weeks in the intervention by Pasagian-Macaulay et al. (48) were not associated with a change in 2-OHE1 or 16α-OHE1. The suggestion is that modest body mass loss or small changes in body composition may not be sufficient to impact estrogen metabolites (47).

An inverse association between body fat and 2:16α-OHE1 was noted at baseline and post-intervention, while a change in body fat was not associated with a change in 2:16α-OHE1. In addition, a positive association with change total lean mass was noted in the present study. An association between body fat and 2:16α-OHE1 is consistent across much of the literature (25, 26, 28, 54-56), however an association with increases in lean body mass is more novel. Atkinson et al. (47) also showed a positive association between lean body mass and 2-OHE1 in the exercise group. In the present study, an association between increase in lean body mass and increase in 2:16α-OHE1 was not accounted for by a possible related reduction in percent body fat. A mechanism linking lean body mass to estrogen metabolism has not been identified.

Lean body mass is a major player in insulin stimulated glucose uptake and storage, and therefore overall energy balance, through a variety of signally pathways. As a result, a significant positive impact of physical activity and lean body mass on risk of cardiovascular disease and metabolic conditions (i.e. insulin resistance) has been reported (58). In addition, a number of these signally pathways are metabolic hormones that have been proposed to impact cancer risk (1). It is possible that lean body mass may impact estrogen metabolism through an interaction with a variety of metabolic hormones and growth factors, however, such an association is speculative and the mechanisms involved unknown.

The pattern of body fat may also have a role to play in estrogen metabolism. The present study found an inverse association between body fat in the hip and abdominal regions and 2:16α-OHE1. Overall, body fat levels and the distribution of body fat have not been measured well in the previous literature on estrogen metabolism. Skin folds (24, 25, 29), bioelectrical impedance (26), BMI (27, 28, 48), and WHR (28, 48) have been used. Using DEXA, the current gold standard for body composition measurement, a weak inverse relationship between increase in intra-abdominal fat and decrease in 2-OHE1 was seen in the control group by Atkinson et al. (47) in postmenopausal women. These authors note that the reasons for this association are unclear but that the low levels of aromatase activity in the intra-abdominal body fat may result in lower levels of estrogens available for conversion to 2-OHE1 and 16α-OHE1 (47).

The majority of research on estrogen metabolites 2-OHE1 and 16α-OHE1 has used a monoclonal antibody enzyme-linked immunoassay, Estramet™ produced by Immuna Care Corporation. This assay has shown good agreement with gas chromatography mass spectroscopy (50, 51) and reported intra- and inter-assay variation for 2-OHE1 and 16α-OHE1 that range from 4.0-12.0% (39, 41, 48). Samples stored at -80°C for more than a year have also shown good stability over time (41). The variability of estrogen metabolism within an individual over time is another important consideration. A considerable range in values across individuals and significant within person variability has been noted. Chen et al. (59) reported moderate variability of within-person 2-OHE1 and 16α-OHE1 levels (25-50%) in premenopausal women not using oral contraceptives. A high correlation between 2:16α-OHE1 in a single urine sample and an average of samples over eight weeks has also been shown (r=.85), however, collection was not standardized by day of the menstrual cycle (59). In postmenopausal women, Rinaldi et al. (60) reported that intra-class correlations (ICC) for three urine samples over several years (between 0.9 and 7.7 years) were low for 2-OHE1 (ICC = 0.32) and 16α-OHE1 (ICC = 0.26) and 2:16α-OHE1 (ICC = 0.18) and reliability of the measure overall was "unsatisfactory" for a prospective study on breast cancer risk factors and sex steroid concentrations.

The menstrual cycle also appears to significantly impact estrogen metabolites, which suggests the importance of identifying the day of sampling in relation to phase of the menstrual cycle. Higher absolute values of 2-OHE1 and 16α-OHE1, and higher 2:16α-OHE1, have been reported in the periovulatory and midluteal phases of the menstrual cycle, following the pattern of estradiol and estrone (29, 40, 42). Westerlind et al. (41) also noted higher absolute levels of 2-OHE1 and 16α-OHE1 in the luteal compared to the follicular phase, but no significant difference in 2:16α-OHE1 across the menstrual cycle. Xu et al. (42) proposed that the difference in estrogen metabolites levels across the menstrual cycle are due to the associated hormone fluctuations, which may in turn modulate hepatic enzyme activities of CYP1A2 and CYP3A4, the enzymes that catalyze 2- and 16α-hydroxylation. In the present study, determination of mid-luteal phase of the menstrual cycle phase was only estimated from length of previous menstrual cycle and confirmed retrospectively by average salivary progesterone levels. Therefore, the timing of urine collection used to determine estrogen metabolites may not have captured the mid-luteal phase for all participants and this may have influenced the results of the present study.

Menstrual dysfunction observed with high intensity training in young athletes has been associated with higher 2-hydroxylation in swimmers and varsity rowers, compared to similar athletes who did not develop menstrual dysfunction (24-26). Eligibility criteria for the present study included guidelines that defined normal menstrual cycle length and the number of menstrual cycles in the past year in an effort to avoid individuals with a history of menstrual dysfunction. However, verification of the ovulatory status of participants was a limitation of the study protocol. The use of three mid-luteal salivary samples is only a proxy measure of ovulation, and may not adequately capture subtle changes in luteal phase length. More frequent sampling or methods to capture the luteinizing hormone surge which accompanies ovulation would have provided additional information about the menstrual cycles of participants but would have been more burdensome to participants and costly. Therefore, the ability to determine the possible impact of menstrual dysfunction on 2-OHE1 and 16α-OHE1 and higher 2:16α-OHE1 was limited in this study.

An additional explanation for the null findings in the present study is that a true association may not have been detected due to design or methodological reasons. The present study design was aimed to increase aerobic fitness in the exercise group over 12 weeks and examine the effect of such an intervention on estrogen metabolites. The type of physical activity intervention chosen had a direct impact on fitness and body composition outcomes but not on estrogen metabolites. Methodological issues surrounding the population selected, sampling methods, measurement methods and statistical analysis all had a potential impact on the findings of the present study and the other intervention studies.

The small sample size of the study could have contributed to the present null findings. Firstly, the overall within person variability, variability of across the menstrual cycle and variability noted across individuals may have limited the ability to determine an overall effect of the intervention on estrogen metabolism. Secondly, the sample size limited the ability to examine the contribution of participant characteristics, beyond changes in aerobic fitness, to estrogen metabolites levels (i.e. subgroup analyses). However, in the present study the direction of change in 2-OHE1, 16α-OHE1 and 2:16α-OHE1 are uniformly moving in an unfavourable direction based on the literature on estrogen metabolism and physical activity, similar to the findings in a study of 170 postmenopausal women by Atkinson et al. (47). This suggests that a larger sample size, while perhaps alleviating the impact of the large variability of these measures, may not change the direction of the present findings.

The acute effect of physical activity is another factor which may have impacted the 2-OHE1 and 16α-OHE1 values reported in this study. Participants were asked to avoid exercise for 24 hours prior to urine sampling in an effort to eliminate a possible contribution of acute short-term changes associated with an acute bout of activity. A bout of physical activity may impact various hormone levels (i.e. cortisol) for the following 24-48 hours or even longer (46). The acute effect of physical activity on estrogen metabolites has been examined by De Cree et al. (61, 62). No change in 2-OHE1 levels from baseline was noted in serum samples taken prior to the start of an maximal incremental exercise test and at highest stage reached, while 16α-OHE1 was not measured. However, 2-OHE1 has not been measured over a longer time course following acute exercise (i.e. next several hours or days) to determine the implications for appropriate timing of samples to reflect a "resting" or usual value.

The type of exercise intervention chosen may also have impacted the findings. The intervention was designed to improve aerobic fitness, measured as VO_{2max}, in a short period of time. The various aspects of exercise prescription, namely frequency, intensity, type and time may not have been ideal to alter estrogen metabolism. While improvement in aerobic fitness is an objective measure of exercise training, the associations of estrogen metabolites with body composition in this study and several others suggests that an exercise intervention should perhaps focus on reducing body fat or increasing lean body mass rather than aiming to improve aerobic fitness as the primary mechanism of change. Snow et al. (26) found a higher 2-OHE1 level in those who developed menstrual dysfunction compared to those who did not, in a high intensity phase of a varsity rowing, and overall 2-OHE1 was positively associated with degree of leanness. During the intense training phase, both groups of athletes lost body mass and increased leanness to a similar degree and nutrient intake did not vary between the two groups or across training phases of different intensities. The development of menstrual dysfunction, along with a similar calorie intake across training phases of differing intensity, suggests that negative energy balance, the currently proposed mechanism for menstrual dysfunction (63, 64), may be a key factor in altering estrogen metabolism. The findings of Russell et al. (24,

25) also support an association between a combination of high intensity training and menstrual dysfunction with alterations in estrogen metabolite levels. Overall the evidence suggests that an intervention achieving negative energy balance through dietary intake, physical activity or a combination of the two may be more likely to alter estrogen metabolites. These data suggest that total volume of physical activity may be the key factor for altering estrogen metabolism.

However, less intense exercise has also been associated with changes in estrogen metabolism. Bentz et al. (27) noted an association between MET-hours per day of physical activity, with the highest 2:16α-OHE1 seen in the highest quartile of physical activity, which averaged 7.2 MET-hours per day. Matthews et al. (28) also found an association with leisure time physical activity greater or equal to 0.5 MET-hours per day, with the median values from 1.0 to 2.0 MET-hours per day, which is equivalent to 15-30 minutes per day of brisk walking.

The optimal timing and length of the intervention may have also contributed to the null findings in the present study. The effects of a short physical activity intervention on estrogen metabolism may not mimic the effect of more habitual patterns of physical activity and energy balance over a lifetime. One study has examined the impact of lifetime physical activity on estrogen metabolite levels. Matthews et al. (28) interviewed 109 Chinese women living in Shanghai about their lifetime occupation activity and non-occupational physical activity, which included household, exercise and sports and transportation, in adolescence (13-19 years) and in adulthood (the 10 years before entering a larger case-control study from which they were sampled). The authors found that those reporting regular exercise (i.e. greater or equal to 0.5 MET-hours per day) had a higher 2:16α-OHE1 than inactive women regardless of BMI, if BMI was >20.

In addition, timing of an intervention over the life span may be an important consideration. It is not known if age or reproductive age have an impact on possible alterations in estrogen metabolism. The early research which reported alteration in estrogen metabolites with high levels of physical activity and menstrual dysfunction, focused on adolescent and young women. It could be that changes in estrogen metabolism are more inducible during this time of life. For postmenopausal women, absolute levels of estrogen metabolites are lower (41), however the impact of menopause on an individual's pattern of estrogen metabolism is unknown. In terms of breast cancer risk, there is a strong positive association between greater physical activity and reduced risk of breast cancer in postmenopausal women, through body mass maintenance, body mass loss and reduction in adiposity (8), suggesting the need for interventions to examine the effects of body mass loss on estrogen metabolites. The association between physical activity and breast cancer in premenopausal women is not as clear. A higher body mass is thought to have a protective effect in premenopausal women, but physical activity can still influence other factors associated with high body mass, such as insulin resistance, sex hormone binding globulin levels, and markers of inflammation that have also been linked to breast cancer risk (8).

It is important to acknowledge the strengths and limitations of this trial. The strengths of this trial include the randomized controlled design, validated measurement (60) of maximal oxygen consumption, individualized exercise programs based on metabolic measures of exercise intensity, supervised aerobic exercise training intervention, a high adherence rate to the intervention, body composition measures by dual-energy x-ray absorptiometry (DEXA), and no loss to follow up.

The limitations of this trial include difficulty standardizing urine sample collection across the menstrual cycle, poor measurement of menstrual dysfunction, variability of estrogen metabolites both within an individual and across individuals, and the small sample size. Accurate determination of menstrual cycle phase requires intensive monitoring of hormonal fluctuations which is difficult to achieve in free-living individuals over a significant length of time (i.e. a 4 month intervention). In addition proxy measures to identify menstrual dysfunction may not provide enough information to determine the alterations along the continuum of menstrual dysfunction (65). The variability in estrogen metabolites across menstrual cycles within an individual makes determination of an intervention related change difficult. While longer sampling to achieve a true baseline for an individual may improve the ability to detect a meaningful change, participant recruitment may then be a challenge and compliance to the trial may be negatively affected. Due to the variability of the measures of estrogen metabolites, a larger sample may be needed in order to observe meaningful group effects if rigorous methods surrounding urine sampling and intervention delivery can be maintained.

Future investigations should focus on the overall association between body composition and estrogen metabolism, and on the impact of changes in body composition on changes in estrogen metabolism. The impact of physical activity could be considered further as part of an intervention to induce negative energy balance and/or change body composition. In addition, further evidence on the impact of usual physical activity, using prospective or objective measures of physical activity in large observational studies, and interventions at different life stages should be undertaken to follow up on recently observed associations, and to determine if time of life has an impact on ability of physical activity to alter estrogen metabolites. In addition, the findings of the present study suggest that a resistance exercise intervention to increase lean body mass may alter estrogen metabolism. A further consideration is improved documentation of usual estrogen metabolite levels and within person variability in order to better understand the results of intervention studies.

In summary, the present study examined the effect of an aerobic exercise intervention, designed to improve maximal aerobic fitness, on estrogen metabolite 2-OHE1, 16α-OHE1, and their ratio, 2:16α-OHE1 in premenopausal women. Higher 2-OHE1 and higher 2:16α-OHE1 have been linked to health conditions, most notably breast cancer risk, and physical activity has been suggested to favourably alter the estrogen metabolism. Thirty-two premenopausal women, who met the eligibility criteria which controlled for many additional factors that have been association with estrogen metabolites, and who were sedentary or recreationally active, were randomized. Seventeen women were randomly assigned to a 12-week supervised exercise intervention and 15 women were randomly assigned to maintain their usual lifestyle for 12 weeks. Urinary estrogen metabolite pattern was compared over the intervention in samples collected in the luteal phase of four menstrual cycles. No change in estrogen metabolism pattern was noted with the intervention, despite a significant increase in aerobic fitness in the exercise group. In conclusion, a 12-week aerobic exercise intervention in premenopausal women, which resulted in significant changes in aerobic fitness and body fat, did not significantly change urinary excretion of 2-OHE1, 16α-

OHE1 or 2:16α-OHE1, proposed biomarkers of breast cancer risk. Baseline and post intervention body fat were favourably associated with estrogen metabolite levels. In addition, a positive association between an increase in lean body mass and higher 2:16α-OHE1 was seen. Future research should focus on the impact of body composition on estrogen metabolites, and further investigations into the impact of physical activity should examine the impact of negative energy balance and resistance training.

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1. Table 1. Baseline characteristics of randomized participants (n=32).

	Overall (n=32)	Exercise (n=17)	Control (n=15)	p
Demographics				
Age, Mean± SE, y	25.7 ± 0.9	25.5 ± 1.1	25.9 ± 1.3	.974
Married/Common-law, No. (%)	11 (34%)	6 (35%)	5 (33 %)	.519
Completed Post Secondary Degree, No. (%)	19 (59%)	11 (65%)	8 (54%)	.379
Body composition, Mean ± SE				
Height, cm	168.0 ± 0.9	167.7 ± 1.4	168.4 ± 1.2	.588
Body mass, kg	64.7 ± 1.5	64.1 ± 1.8	65.5 ± 2.5	.831
BMI, kg/m^2	22.9 ± 0.5	22.9 ± 0.7	23.0 ± 0.7	.862
Waist circumference, cm	74.3 ± 1.2	73.9 ± 1.4	74.9 ± 2.1	.860
Hip circumference, cm	100.3 ± 1.1	100.1 ± 1.3	100.5 ± 1.9	.997
WHR	0.74 ± 0.01	0.74 ± 0.01	0.74 ± 0.01	.821
Total body fat, %	32.8 ± 0.7	33.8 ± 1.6	32.1 ± 1.5	.320
Physical activity and fitness, Mean ± SE				
Strenuous exercise, min/week	44.1 ± 9.0	32.4 ± 8.8	57.3 ± 16.1	.186
Moderate exercise, min/week	56.9 ± 9.5	62.1 ±13.7	51.0 ± 13.3	.502
Total Exercise, MET-hours/week	11.5 ± 1.5	9.4 ± 1.5	13.8 ± 2.6	.179
Relative VO$_2$max, ml/kg/min	32.8 ± 0.7	32.8 ± 1.1	32.7 ± 1.0	.979
Absolute VO$_2$max, L/min	2.11 ± 0.06	2.09 ± 0.07	2.13 ± 0.09	.828
Maximum power output, Watts	185 ± 5	184 ± 6	187 ± 9	.966
Respiratory Exchange Ratio	1.33 ± 0.02	1.34 ± 0.02	1.32 ± 0.02	.807
Reproductive function				
Age of menarche, Mean ± SE, y (n=15)	13.0 ± 0.2	12.9 ± 0.2	13.0 ± 0.4	1.00
Reproductive age, Mean ± SE, y (n=15)	12.9 ± 0.9	12.8 ± 1.1	12.9 ± 1.4	.882
Menstrual Cycle length, Mean ±SE, d	29 ± 1	30 ± 1	28 ± 1	.263
Nulliparous, No. (%)	26 (81%)	16 (94%)	10 (66%)	.056

Legend: Reproductive age, age at menarche subtracted from current age; Total exercise, sum of strenuous, moderate and mild minutes of physical activity.

Abbreviations: SE, standard error; BMI, body mass index; WHR, waist-to-hip ratio; MET, metabolic equivalent (measure of energy cost of activity); VO$_2$max, maximal oxygen consumption measured during a graded incremental exercise test on a cycle ergometer.

Table 2 – Effects of aerobic exercise training on untransformed (and log transformed) estrogen metabolism in premenopausal women (N=32)

	Cycle 1	p for baseline difference	Cycle 2	Cycle 3	Cycle 4	Group (2) x Time (4) repeated measures		Mean Change from cycle 1 to cycle 4	Differences between groups in mean change	p for between group difference
	Mean ± SE		Mean ± SE	Mean ± SE	Mean ± SE	F	p	M [95% CI]		
2-OHE1										
Exercise	31.7 ± 7.4	.985	25.4 ± 3.0	27.6 ± 3.6	31.6 ± 4.0	.03	.860	-0.1 [-12.7 to 12.5]		.835
Control	27.6 ± 5.2	(.558)	28.9 ± 4.0	23.5 ± 3.4	28.5 ± 4.1	(.24)	(.627)	1.0 [-11.3 to 13.2]	-1.1 [-18.1 to 15.8]	(.537)
16α-OHE1										
Exercise	18.9 ± 4.1	.462	17.2 ± 2.4	18.7 ± 3.9	21.8 ± 4.1	.00	.962	2.9 [-6.3 to 12.2]		.806
Control	13.6 ± 1.5	(.395)	16.4 ± 2.5	16.9 ± 3.0	17.2 ± 2.2	(.07)	(.797)	3.5 [-1.5 to 8.5]	-0.6 [-11.1 to 9.9]	(.811)
Total EMC										
Exercise	50.6 ± 11.3	.720	42.6 ± 4.2	46.3 ± 6.8	53.4 ± 7.1	.01	.930	2.8 [-18.2 to 23.9]		.925
Control	41.2 ± 6.3	(.491)	45.3 ± 6.0	40.4 ± 6.1	45.7 ± 5.9	(.00)	(.987)	4.5 [-11.7 to 20.6]	-1.7 [-27.7 to 24.4]	(.788)
2:16α-OHE1										
Exercise	1.78 ± 0.20	.865	1.72 ± 0.21	1.73 ± 0.17	1.70 ± 0.19	.54	.466	-0.08 [-0.45 to 0.29]		.925
Control	1.90 ± 0.28	(.991)	2.05 ± 0.29	1.57 ± 0.19	1.74± 0.16	(.00)	(.987)	-0.16 [-0.69 to 0.38]	-0.07 [-0.69 to 0.54]	(.611)

Exercise group, n=17; Control group, n=15
Legend: 2-OHE1, 16α-OHE1 and total estrogen metabolite concentration presented in ng/ml/mg Cr. Mean change is the cycle 1 value subtracted from the cycle 4 value. Difference between groups is a comparison of mean change between groups from cycle 1 to cycle 4.
Abbreviations: Total EMC, Total estrogen metabolite concentration (addition of 2-OHE1 and 16α-OHE1).

Table 3 – Associations between baseline untransformed and (log transformed) luteal estrogen metabolites and self-reported exercise, aerobic fitness, and body mass and composition in premenopausal women (N=32)

	2:16α-OHE1		2-OHE1		16α-OHE1	
	r value	p value	r value	p value	r value	p value
Demographic and reproductive						
Age	-.17 (-.13)	.363 (.492)	-.22 (-.17)	.222 (.365)	-.14 (-.12)	.444 (.512)
Reproductive age (n=15)	-.18 (-.20)	.331 (.290)	-.22 (-.19)	.232 (.315)	-.17 (-.10)	.376 (.599)
Body composition						
Height, cm	.17 (.17)	.352 (.345)	.03 (.04)	.857 (.843)	-.10 (-.11)	.598 (.560)
Body mass, kg	-.10 (-.16)	.591 (.386)	.03 (.02)	.853 (.895)	.18 (.18)	.332 (.321)
BMI, kg/m^2	-.21 (-.27)	.252 (.134)	-.01 (.00)	.977 (.990)	.20 (.25)	.272 (.163)
Waist circumference, cm	-.24 (-.31)	.196 (.083)	-.01 (-.05)	.952 (.787)	.19 (.22)	.295 (.236)
Hip circumference, cm	-.11 (-.16)	.540 (.382)	-.02 (-.04)	.904 (.820)	.06 (.09)	.732 (.633)
WHR	-.31 (-.34)	.082 (.055)	-.02 (-.04)	.932 (.844)	.26 (.27)	.157 (.143)
Total body fat, %	-.30 (-.40)	.099 (.044)	.00 (.00)	.995 (.992)	.32 (.33)	.078 (.067)
Total body fat, kg	-.25 (-.31)	.163 (.089)	-.02 (.02)	.927 (.910)	.24 (.31)	.187 (.083)
Total lean mass, kg	.29 (.14)	.109 (.454)	.13 (.04)	.484 (.846)	-.08 (-.08)	.684 (.682)
Gynoid body fat, %	-.30 (-.39)	.096 (.028)	-.07 (-.11)	.719 (.543)	.23 (.20)	.212 (.278)
Gynoid body fat, kg	-.17 (-.28)	.354 (.115)	-.05 (-.08)	.773 (.648)	.13 (.14)	.478 (.438)
Android body fat, %	-.28 (-.36)	.116 (.044)	.00 (.04)	.990 (.847)	.30 (.38)	.097 (.031)
Android body fat, kg	-.21 (-.30)	.239 (.092)	.05 (.11)	.789 (.556)	.31 (.43)	.087 (.013)
Physical activity and fitness						
Strenuous exercise, min/week	.38 (.30)	.034 (.102)	.11 (.05)	.534 (.770)	-.11 (-.20)	.555 (.286)
Moderate exercise, min/week	-.09 (-.08)	.641 (.671)	-.17 (-.05)	.362 (.793)	-.01 (.00)	.939 (.987)
Total Exercise, MET-hours/week	.13 (.13)	.486 (.467)	-.03 (-.04)	.891 (.827)	-.02 (-.18)	.915 (.324)
Relative VO_2max, ml/kg/min	.26 (.27)	.154 (.140)	.06 (.02)	.738 (.921)	-.15 (-.22)	.417 (.226)
Absolute VO_2max, L/min	.16 (.09)	.385 (.638)	.05 (.03)	.779 (.880)	-.05 (-.04)	.772 (.827)

Legend: Reproductive age, age at menarche subtracted from current age; Abbreviations: SE, standard error; BMI, body mass index; WHR, waist-to-hip ratio; MET, metabolic equivalent (measure of energy cost of activity); VO_2max, maximal oxygen consumption measured during a graded incremental exercise test.

Table 4 – Adherence to the 12-week exercise intervention (Frequency)

	Overall (44 sessions)	Week 1-4 (12 sessions)	Week 5-8 (16 sessions)	Week 9-12 (16 sessions)
All Exercisers (N=17)				
Adherence, Mean ± SE	39.4 ± 1.3	11.5 ± 0.3	14.9 ± 0.6	13.0 ± 1.1
Adherence, %	91	96	93	81
Range,%	64-100	75-100	88-100	13-100
Adherence ≥80%, No., (%)	14 (82)	15 (88)	16 (94)	12 (71)

Abbreviations: SE, standard error.

Table 5 - Adherence to the 12-week exercise intervention (Duration and Intensity)

	Week 1-4	Week 5-8			Week 9-12		
	Base	Base	Interval 1	Interval 2	Base	Interval 1	Interval 2
Exercise Prescription, Mean±SE							
Duration completed, min/week	104.5 ± 2.9	73.5 ± 3.1	19.6 ± 0.4	19.5 ± 1.3	60.4 ± 4.8	17.7 ± 1.0	17.1 ± 1.3
RPE (6-20)	13.2 ± 0.2	13.2 ± 0.2	15.2 ± 0.3	17.4 ± 0.3	13.6 ± 0.2	15.5 ± 0.3	19.3 ± 0.1
Power output, Watts	87 ± 3	93 ± 3	114 ± 3	185 ± 5	105 ± 3	124 ± 4	193 ± 5
% Maximal heart rate*	80.3 ± 0.8	79.9 ± 0.9	85.9 ± 0.8	90.5 ± 0.8	80.0 ± 1.0	86.9 ± 1.0	99.2 ± 0.6
% Maximal power output*	47.7 ± 1.3	51.0 ± 1.3	62.5 ± 1.4	100.7 ± 1.2	57.5 ± 1.3	68.1 ± 1.9	105.6 ± 1.8

Legend: Base, base aerobic training at wattage corresponding to approximately 25% higher than VE/VO_2; *Interval 1*, two 10-minute intervals at a power output corresponding to VE/VCO_2 with 10 minutes with 10-minutes of easy cycling between; *Interval 2*, intervals at a power output corresponding to VO_2max for 30 seconds followed by 30 seconds of easy pedaling 20 times (Week 5-8) or two minutes at a power output corresponding to VO_2max and three minutes of easy pedaling, four times (Week 9-12). Duration of *Interval 1* determined as amount of time at specified power output (not counting "rest or easy pedaling between intervals) and for *Interval 2* determined as time spent during entire interval session (includes both interval and associated "rest" or easy pedaling).
* as determined from incremental graded exercise test at baseline. Should add % meeting cut point similar to frequency (if it makes sense)

Abbreviations: SE, standard error; RPE, rating of perceived exertion, which is based on a scale from 6-20 (45), where 13 is "somewhat hard", 15 is "hard", 17 is "very hard" and 19 is "very, very hard".

Table 6- Effects of aerobic exercise training on aerobic fitness in premenopausal women (N=32)

	Baseline	p for baseline difference	Midpoint	Post Intervention	Mean Change from baseline to post-intervention	Differences between groups in mean change	p for between group differences
	M (SE)		M (SE)	M (SE)	M [95% CI]	M [95% CI]	
VO_{2max}, ml/kg/min							
Exercise	32.8 ±1.1		35.0 ± 1.2	37.4 ± 1.1	4.6 [3.2 to 6.0]		
Control	32.7 ± 1.0	.979	NA	31.8 ± 1.1	-1.0 [-1.9 to 0.0]	5.6 [3.7 to 7.2]	< 0.001
VO_{2max}, L/min							
Exercise	2.09 ± 0.07		2.25 ± 0.07	2.37 ± 0.06	0.27 [0.17 to 0.38]		
Control	2.13 ± 0.09	.828	NA	2.08 ± 0.08	-0.06 [-0.13 to 0.01]	0.33 [0.20 to 0.45]	< 0.001
Power output at VO_{2max}, Watts							
Exercise	184 ± 6		207 ± 5	221 ± 5	37 [31 to 43]		
Control	187 ± 9	.807	NA	186 ± 8	-1 [-11 to 10]	37 [26 to 48]	< 0.001
Heart rate at VO_{2max}, bpm							
Exercise	187 ± 2		185 ± 2	188 ± 2	1 [-1 to 3]		
Control	185 ± 3	.464	NA	184 ± 3	0 [-4 to 2]	2 [-1 to 5]	.242
RER							
Exercise	1.35 ± 0.02		1.32 ± 0.02	1.33 ± 0.02	0.01 [-0.04 to 0.05]		
Control	1.34 ± 0.02	.966	NA	1.33 ± 0.02	-0.01 [-0.07 to 0.04]	0.02 [-0.05 to 0.09]	.530
VO_2 at V_E/VO_2, L/min							
Exercise	1.01 ± 0.04		1.16 ± 0.05	1.26 ± 0.05	0.25 [0.17 to 0.32]		
Control	1.01 ± 0.05	.972	NA	1.03 ± 0.05	0.02 [-0.09 to 0.13]	0.22 [0.10 to 0.35]	.001
Power output Ve/VO_2, Watts							
Exercise	67 ± 4		80 ± 4	91 ± 5	24[16 to 32]		
Control	65 ± 5	.692	NA	66 ± 4	1 [-6 to 8]	23 [12 to 34]	<0.001
VO_2 at V_E/VCO_2, L/min							
Exercise	1.37 ± 0.04		1.49 ± 0.06	1.59 ± 0.04	0.22 [0.14 to 0.31]		
Control	1.34 ± 0.05	.685	NA	1.35 ± 0.04	0.01 [-0.08 to 0.11]	0.21 [0.09 to 0.33]	.001

Table 6 – continued

	Baseline	p for baseline difference	Midpoint	Post Intervention	Mean Change from baseline to post-intervention	Differences between groups in mean change	p for between group differences
	M (SE)		M (SE)	M (SE)	M [95% CI]	M [95% CI]	
Power output at V_E/VCO_2, Watts							
Exercise	107 ± 5		118 ± 4	125 ± 4	18 [7 to 28]		
Control	104 ± 4	.586	NA	99 ± 5	-5 [-12 to 3]	22 [10 to 34]	.001

Exercise group, n=17; Control group, n=15

Legend: Mean change is the baseline value subtracted from the post-intervention value. Difference between groups is a comparison of mean change between groups.

Abbreviations: VO_2max, maximal oxygen consumption measured during a graded incremental exercise test on a cycle ergometer; V_E/VO_2, ventilatory equivalent for oxygen; V_E/VCO_2, ventilatory equivalent for carbon dioxide.

Table 7 - Effects of aerobic exercise training on body mass and composition in premenopausal women (N=32)

	Baseline	p for baseline differences	Midpoint	Post Intervention	Mean Change	Differences between groups in mean change	p for between group differences
	M ± SE		M ± SE	M ± SE	M [95% CI]	M [95% CI]	
Body mass (kg)							
Exercise	64.1 ± 1.8		64.1 ± 1.8	63.7 ± 1.8	-0.4 [-1.4 to 0.7]		
Control	65.5 ± 2.5	.657	65.6 ± 3.1	66.0 ± 2.6	0.5 [-0.4 to 1.4]	-0.9 [-2.2 to 0.5]	.209
BMI (kg/m^2)							
Exercise	22.9 ± 0.7		22.8 ± 0.6	22.7 ± 0.6	-0.2 [-0.5 to 0.2]		
Control	23.0 ± 0.7	.875	23.2 ± 0.9	23.2 ± 0.8	0.2 [-0.1 to 0.5]	-0.4 [-0.8 to 0.1]	.146
Waist Circumference (cm)							
Exercise	73.9 ± 1.4		72.4 ± 1.2	71.9 ± 1.4	-2.0 [-3.6 to -0.4]		
Control	74.9 ± 2.1	.677	73.1 ± 2.2	73.3 ± 1.8	-1.6 [-3.1 to -0.2]	-0.4 [-2.5 to 1.8]	.736
Hip Circumference (cm)							
Exercise	100.1 ± 1.3		100.2 ± 1.2	99.5 ± 1.4	-0.6 [-1.7 to 0.4]		
Control	100.5 ± 1.9	.849	101.1 ± 2.5	100.6 ± 2.0	0.0 [-1.2 to 1.3]	-0.6 [-2.2 to 0.9]	.407
WHR							
Exercise	0.74 ± 0.01		0.72 ± 0.01	0.72 ± 0.01	-0.02 [-0.03 to 0.00]		
Control	0.74 ± 0.01	.668	0.72 ± 0.01	0.73 ± 0.01	-0.02 [-0.03 to 0.00]	0.00 [-0.02 to 0.02]	.938
% Body Fat							
Exercise	33.8 ± 1.6		NA	33.0 ± 1.6	-0.9 [-1.8 to 0.1]		
Control	32.1 ± 1.5	.441	NA	32.9 ± 1.5	0.8 [0.1 to 1.5]	-1.7 [-2.8 to -0.5]	.006
Total Fat Mass (kg)							
Exercise	20.9 ± 1.6		NA	20.2 ± 1.5	-0.6 [-1.5 to 0.2]		
Control	20.1 ± 1.4	.736	NA	20.8 ± 1.5	0.6 [0.0 to 1.2]	-1.2 [-2.2 to -0.2]	.018
Total Lean Mass (kg)							
Exercise	39.7 ± 0.7		NA	40.2 ± 0.7	0.5 [0.0 to 1.0]		
Control	42.0 ± 1.5	.167	NA	41.5 ± 1.4	-0.4[-0.9 to 0.0]	0.9 [0.2 to 1.6]	.009
Gynoid Fat Mass (%)							
Exercise	43.8 ± 1.19		NA	43.0 ± 1.2	-0.8 [-1.7 to 0.1]		
Control	41.9 ± 1.16	.256	NA	42.7 ± 1.2	0.8 [-0.2 to 1.8]	-1.6 [-3.0 to -0.4]	.014
Gynoid Fat Mass (kg)							
Exercise	4.8 ± 0.3		NA	4.7 ± 0.3	-0.1 [-0.3 to 0.1]		
Control	4.7 ± 0.3	.790	NA	4.8 ± 0.3	0.2 [0.0 to 0.3]	-0.3 [-0.5 to -0.1]	.014

Table 7 – continued

	Baseline M ± SE	p for baseline differences	Midpoint M ± SE	Post Intervention M ± SE	Mean Change M [95% CI]	Differences between groups in mean change M [95% CI]	p for between group differences
Android Fat Mass (%)							
Exercise	35.7 ± 2.2		NA	34.7 ± 2.1	-1.0 [-2.6 to 0.6]		
Control	34.3 ± 2.1	.669	NA	34.7 ± 2.0	0.4 [-0.6 to 1.4]	-1.4 [-3.3 to 0.5]	.149
Android Fat Mass (kg)							
Exercise	1.6 ± 0.2		NA	1.5 ± 0.2	-0.1 [-0.2 to 0.0]		
Control	1.5 ± 0.1	.832	NA	1.5 ± 0.1	0.0 [0.0 to 0.1]	-0.1 [-0.2 to 0.0]	.086

Exercise group, n=16; Control group, n=15
Legend: Mean change is the baseline value subtracted from the post-intervention value. Difference between groups is a comparison of mean change between groups.
Abbreviations: BMI, body mass index;WHR, waist-to-hip ratio

Table 8 - Baseline dietary intake of randomized participants (N=30).

	Baseline	p for baseline difference	Post Intervention	Mean Change from baseline to post-intervention	Differences between groups in mean change	p for between group differences
	M ± SE		M ± SE	M [95% CI]	M [95% CI]	
Calories, kcal						
Exercise	1876 ± 116		1729 ± 107	-147 [-396 to 103]		
Control	1919 ± 133	.979	1915 ± 165	-4 [-204 to 196]	-143 [-180 to 452]	.371
Total Fat, g						
Exercise	68 ± 4		62 ± 5	-6 [-17 to 5]		
Control	71 ± 8	.828	76 ± 12	5 [-10 to 21]	-12 [-29 to 6]	.183
Protein, g						
Exercise	81 ± 10		72 ± 8	-10 [-28 to 9]		
Control	86 ± 7	.807	78 ± 9	-9 [-20 to 3]	-1 [-20 to 23]	.940
Carbohydrates, g						
Exercise	237 ± 16		229 ± 59	-8 [-42 to 26]		
Control	241 ± 15	.966	234 ± 12	-6 [-31 to 19]	-2 [-45 to 42]	.946
Dietary fibre, g						
Exercise	20 ± 2		18 ± 2	-2 [-6 to 2]		
Control	22 ± 3	.584	20 ± 9	-2 [-6 to 3]	0 [-5 to 6]	.887
Fat:Fibre ratio						
Exercise	4.1 ± 0.4		3.9 ± 0.3	-0.2 [-1.1 to 0.7]		
Control	3.7 ± 0.4	.414	4.1 ± 0.5	0.4 [-0.3 to 1.1]	-0.6 [-1.8 to 0.6]	.290

Exercise group N=16; Control group N=12
Includes participants with pre- and postintervention data.

Table 9 - Associations between changes in untransformed (and log transformed) estrogen metabolites and changes in aerobic fitness/body composition from baseline to postintervention in premenopausal women (N=32)

	Change in 2:16α-OHE1		Change in 2-OHE1		Change in 16α-OHE1	
	r value	p value	r value	p value	r value	p value
Change in body mass and composition						
Body mass, kg	-.10 (-.08)	.581 (.667)	-.14 (-.08)	.452 (.656)	.02 (-.14)	.903 (.453)
BMI, kg/m^2	-.08 (-.06)	.678 (.743)	-.14 (-.08)	.456 (.672)	.01 (-.17)	.965 (.352)
Waist circumference, cm	-.16 (-.12)	.387 (.531)	-.12 (-.24)	.508 (.186)	.01 (-.09)	.975 (.621)
Hip circumference, cm	-.22 (-.22)	.232 (.236)	-.28 (-.11)	.121 (.544)	.02 (-.07)	.898 (.710)
WHR	.05 (.03)	.782 (.878)	.12 (-.18)	.529 (.333)	-.04 (-.08)	.835 (.656)
Total body fat, % (DEXA)	-.33 (-.26)	.048 (.147)	-.01 (-.21)	.632 (.254)	.14 (.05)	.461 (.780)
Total body fat, kg (DEXA)	-.13 (-.13)	.196 (.479)	.03 (-.21)	.862 (.250)	.02 (-.10)	.895 (.592)
Total lean mass, kg (DEXA)	.47 (.43)	.006 (.015)	.02 (.18)	.926 (.320)	-.25 (-.20)	.172 (.272)
Gynoid body fat, %	-.27 (-.24)	.142 (.184)	-.16 (-.27)	.373 (.141)	.09 (-.04)	.614 (.827)
Gynoid body fat, kg	-.26 (-.14)	.151 (.439)	-.19 (-.25)	.296 (.174)	.09 (-.21)	.651 (.256)
Android body fat, %	-.08 (-.10)	.647 (.573)	.09 (-.14)	.636 (.451)	.05 (-.04)	.787 (.847)
Android body fat, kg	-.05 (.01)	.776(.944)	.05 (-.15)	.776 (.405)	.04 (-.20)	.851 (272)
Change in aerobic fitness						
VO_2max, ml/kg/min	-.16 (-.20)	.389 (.276)	-.18 (-.18)	.300 (.319)	-.04 (-.08)	.815 (.685)
VO_2max, L/min	-.11 (-.22)	.568 (.229)	-.26 (-.21)	.154 (.247)	-.13 (-.12)	.491 (.501)

Abbreviations: BMI, body mass index ;WHR, waist-to-hip ratio; DEXA, dual-energy x-ray absorptiometry; MET, metabolic equivalent (measure of energy cost of activity); VO_2max, maximal oxygen consumption measured during a graded incremental exercise test on a cycle ergometer.

Table 10- Associations between postintervention untransformed (and log transformed) luteal estrogen metabolism and aerobic fitness/body composition in premenopausal women (N=32)

	2:16α-OHE1		2-OHE1		16α-OHE1	
	r value	p value	r value	p value	r value	p value
Body mass and composition						
Body mass, kg	-.12 (-.27)	.502 (.131)	-.15 (-.23)	.430 (.211)	.01 (-.02)	.979 (.934)
BMI, kg/m^2	-.27 (-.39)	.138 (.028)	-.08 (-.22)	.684 (.226)	.13 (.09)	.467 (.610)
Waist circumference, cm	-.17 (-.23)	.361 (.199)	-.08 (-.15)	.668 (.416)	.08 (.04)	.664 (.839)
Hip circumference, cm	-.15 (-.27)	.409 (.142)	-.15 (-.22)	.419 (.225)	-.02 (-.01)	.918 (.939)
WHR	-.12 (-.04)	.522 (.832)	-.09 (.02)	.629 (.904)	.18 (.06)	.325 (.750)
Total body fat, %	-.29 (-.40)	.110 (.027)	-.06 (-.18)	.744 (.320)	.15 (.14)	.426 (.450)
Total body fat, kg	-.24 (-.39)	.188 (.028)	-.09 (-.23)	.628 (.198)	.10 (.08)	.587 (.672)
Total lean mass, kg	.16 (.03)	.384 (.885)	-.08 (-.11)	.655 (.561)	-.09 (-.14)	.608 (.439)
Gynoid body fat, %	-.28 (-.36)	.123 (.044)	-.12 (-.21)	.502 (.259)	.06 (.08)	.765 (.651)
Gynoid body fat, kg	-.21 (-.36)	.255 (.045)	-.14 (-.29)	.458 (.114)	.03 (-.01)	.888 (.973)
Android body fat, %	-.29 (-.38)	.114 (.034)	-.10 (-.16)	.586 (.395)	.11 (.15)	.558 (.402)
Android body fat, kg	-.27 (-.35)	.138 (.047)	-.13 (-.17)	.478 (.349)	.08 (.12)	.677 (.523)
Aerobic fitness						
VO_2max, ml/kg/min	.20 (.19)	.263 (.295)	.27 (.18)	.143 (.330)	.05 (.03)	.782 (.863)
VO_2max, L/min	.04 (-.04)	.823 (.813)	.07 (.00)	.696 (.992)	.02 (.04)	.914 (.828)

Abbreviations: BMI, body mass index ;WHR, waist-to-hip ratio; MET, metabolic equivalent (measure of energy cost of activity);VO_2max, maximal oxygen consumption measured during a graded incremental exercise test on a cycle ergometer.

Table 11 - Associations between changes in untransformed (and log transformed) estrogen metabolites and changes in aerobic fitness/body composition from baseline to postintervention in exercise group (N=17)

	2:16α-OHE1		2-OHE1		16α-OHE1	
	r value	p value	r value	p value	r value	p value
Body mass and composition						
Body mass, kg	-.04 (.01)	.881 (.973)	-.31 (-.18)	.226 (.499)	-.06 (.32)	.808 (.210)
BMI, kg/m^2	-.03 (.02)	.903 (.933)	-.32 (-.17)	.218 (.506)	-.08 (-.35)	.775 (.175)
Waist circumference, cm	-.16 (-.18)	.532 (.479)	-.34 (-.44)	.186 (.079)	-.09 (-.26)	.747 (.319)
Hip circumference, cm	-.40 (-.32)	.113 (.210)	-.50 (-.24)	.041 (.351)	.06 (-.07)	.815 (.781)
WHR	.14 (-.10)	.586 (.710)	-.20 (-.47)	.437 (.059)	-.23 (-.29)	.373 (.252)
Total body fat, %	-.29 (-.25)	.258 (.328)	-.18 (-.26)	.498 (.318)	.09 (.00)	.729 (1.00)
Total body fat, kg	-.21 (-.07)	.411 (.803)	-.25 (-.35)	.333 (.165)	-.07 (.23)	.793 (.373)
Total lean mass, kg	.31 (.53)	.220 (.029)	-.12 (-.04)	.646 (.873)	-.22 (-.40)	.400 (.108)
Gynoid body fat, %	-.21 (-.25)	.424 (.327)	-.20 (-.43)	.322 (.087)	.05 (-.11)	.851 (.677)
Gynoid body fat, kg	-.18 (-.02)	.498 (.935)	-.46 (-.49)	.063 (.047)	-.16 (-.48)	.529 (.050)
Android body fat, %	-.21 (-.20)	.428 (.437)	-.15 (-.31)	.567(.230)	-.06 (-.17)	.808 (.507)
Android body fat, kg	-.10 (.02)	.701 (.949)	-.14 (-.36)	.599 (.160)	-.08 (-.43)	.765 (.089)
Aerobic fitness						
VO_2max, ml/kg/min	-.35 (-.38)	.164 (.136)	-.35 (-.25)	.170 (.331)	.11 (-.13)	.687 (.610)
VO_2max, L/min	-.29 (-.38)	.266 (.133)	-.50 (-.34)	.041 (.181)	-.07 (-.26)	.801 (.317)

Abbreviations: BMI, body mass index ;WHR, waist-to-hip ratio; DEXA, dual-energy x-ray absorptiometry; MET, metabolic equivalent (measure of energy cost of activity); VO_2max, maximal oxygen consumption measured during a graded incremental exercise test on a cycle ergometer.

Table 12 - Menstrual cycle length across four consecutive menstrual cycles combined with mid-luteal progestone levels for first and fourth menstrual cycles (N=32).

		Cycle 1				Cycle 2	Cycle 3	Cycle 4				MD
		Saliva Samples						Saliva Samples				
ID		1	2	3	Length	Length	Length	1	2	3	Length	
1	E	.685	.451	.521	29	34	30				30	N
2	E	.283	.	.083	31	33	33	.	.028	.	33	N
3	E	.160	.219	.289	34	23	33	.152	.115	.085	30	Y
4	E	.	.	.149	27	29	29	.	.	.	29	N
6	E	.027	.044	.149	30	25	24	.	.	.	31	N
7	C	.027	.215	.227	24	26	28	.054	.142	.163	28	N
9	C	.271	.302	.193	26	24	26	.353	.138	.191	26	N
10	C	.043	.029	.050	32	33	30	.	.	.186	30	N
11	C	.312	.142	.068	28	27	29	.	.	.	29	N
13	C	.174	.115	.156	28	29	26	.111	.181	.173	28	N
14	C	.212	.172	.183	25	26	28	.232	.242	.058	24	N
15	E	.	.323	.304	26	23	26	.250	.078	.148	28	Y
16	E	.	.131	.134	29	29	30	.140	.143	.146	30	N
18	E	.084	.110	.169	29	27	29	.020	.279	.131	44	Y
19	C	.174	.102	.181	25	25	26	.040	.060	.092	30	Y
20	C				30	28	31				30	N
21	E	.024	.076	.118	31	25	27	.106	.166	.159	27	N
22	E	.141	.240	.195	32	35	38	.158	.086	.	38	N
23	E	.136	.076	.124	27	26	31	.	.	.	30	N
24	C	.073	.042	.095	33	30	28	.	.	.	28	N
26	C	.361	.789	.058	26	25	27	.	.	.	27	N
28	E	.115	.085	.039	34	34	42	.108	.115	.120	47	Y
29	C	.138	.628	.622	33	31	29	.	.	.555	29	N

Table 12 – continued

		Cycle 1			Cycle 2	Cycle 3	Cycle 4					MD
		Saliva Samples						Saliva Samples				
ID		1	2	3	Length	Length	Length	1	2	3	Length	
31	C	.286	.326	.654	35	30	31	.	.	.	31	N
32	E	.242	.450	.569	29	34	40	.600	.351	.412	32	Y
33	C	.161	.181	.216	25	27	31	.188	.038	.142	30	N
34	E	.632	.560	.182	26	27	27	.328	.609	.121	27	N
35	C	.183	.033	.055	24	42	42	.184	.064	.153	26	Y
36	E	.	.	.	27	30	30	.	.	.	30	N
37	E	.210	.164	.199	28	31	29	.104	.102	.345	29	N
38	E	.131	.239	.152	33	32	39	.	.	.	32	Y
40	C	.	.	.	28	29	28	.	.	.	30	N

Legend: Menstrual dysfunction was defined as i) cycles shorter than 24 days, or ii) one cycle longer than 36, or iii) average mid-luteal salivary progesterone values 0.11-0.2 ng/ml (at least two samples available). Cycle length not meeting normal menstrual cycle length (24-36 days) are shaded; Progesterone values not meeting criteria for ovulatory cycle (0.11-0.2 ng/ml), using average of two highest values or single value if only one available, are shaded.
Abbreviations: MD, menstrual dysfunction.

Note: Using Day one as the onset of menses, the mean days of collection were Day 21, 23 and 24 of Cycle 1 and Day 21, 23 and 25 of Cycle 4. To account for the variability in menstrual cycle length, the onset of the following menstrual cycle could also be used as a reference. For these participants this corresponded to mean days of collection of -9, -8, -6 days for Cycle 1 and -10, -8, -6 days for Cycle 4.

Table 13 - Change in luteal 2:16α-OHE1 in the exercise group by selected participant characteristics (N=32)

			Cycle 1	Cycle 4	Mean Change from cycle 1 to cycle 4	Differences between groups in mean change	Group (2) X Factor (2) Interaction
		N	Mean ± SE	Mean ± SE	M [95% CI]	M [95% CI]	p
BMI							.645
<25							
	Exercise	13	2.02 ± 0.20	1.95 ± 0.17	-0.07 [-0.57 to 0.44]		
	Control	12	1.99 ± 0.32	1.81 ± 0.17	-0.18 [-0.85 to 0.49]	0.11 [-0.67 to 0.89]	
>25							
	Exercise	4	0.99 ± 0.27	0.86 ± 0.35	-0.13 [-0.42 to 0.17]		
	Control	3	1.52 ± 0.67	1.47 ± 0.43	-0.06 [-1.72 to 1.61]	-0.07 [-0.95 to 0.81]	
Initial 2:16α-OHE1							.498
<2.00							
	Exercise	11	1.31 ± 0.14	1.38 ± 0.18	0.07 [-0.17 to 0.31]		
	Control	7	0.96 ± 0.13	1.42 ± 0.19	0.46 [-0.11 to 1.04]	-0.39[-0.88 to 0.10]	
≥2.00							
	Exercise	6	2.65 ± 0.21	2.28 ± 0.31	-0.36 [-1.55 to 0.83]		
	Control	8	2.71 ± 0.28	2.0 ± 0.21	-0.69 [-1.45 to 0.06]	0.33 [-.0.85 to 1.52]	
Body mass change							.582
Loss or no change							
	Exercise	8	1.70 ± 0.24	1.81 ± 0.35	0.11 [-0.46 to0.68]		
	Control	9	1.85 ± 0.32	1.60 ± 0.19	-0.14 [-1.51 to 1.22]	0.25 [-.91 to 1.42]	
Gain							
	Exercise	6	2.11 ± 0.59	1.97 ± 0.25	-0.25 [-0.83 to 0.33]		
	Control	9	1.75 ± 0.28	1.59 ± 0.20	-0.16 [-0.74 to 0.42]	-.09 [-.085 to 0.67]	
Change in % body fat							.844
Loss or no change							
	Exercise	13	1.61 ± 0.19	1.68 ± 0.87	0.07 [-0.30 to 0.44]		
	Control	4	1.25 ± 0.46	1.66 ± 0.26	0.41 [-1.32 to 2.15]	-0.34 [-1.24 to 0.55]	
Gain							
	Exercise	4	2.33 ± 0.53	1.76 ± 0.19	-0.57 [-2.1 to 0.91]		
	Control	11	2.13 ± 0.33	1.77 ± 0.20	-.036 [-0.95 to 0.23]	-0.21 [-1.34 to 0.91]	

Table 13 – continued

	N	Cycle 1 Mean ± SE	Cycle 4 Mean ± SE	Mean Change from cycle 1 to cycle 4 M [95% CI]	Differences between groups in mean change M [95% CI]	Group (2) X Factor (2) Interaction p
Change in lean mass (kg)						.262
Gain						
Exercise	11	1.82 ± 0.17	1.96 ± 0.19	0.15 [-0.29 to 0.58]		
Control	6	0.93 ± 0.15	1.52 ± 0.19	0.59 [.00 to 1.18]	-0.45 [-1.12 to 0.23]	
Loss or no change						
Exercise	6	1.72 ± 0.50	1.21 ± 0.33	-0.50 [-1.25 to 0.25]		
Control	9	2.54 ± 0.30	1.89 ± 0.68	-0.65 [-1.31 to 0.01]	0.15 [-0.77 to 1.07]	
Menstrual Dysfunction						.594
Possible						
Exercise	4	1.73 ± 0.59	1.36 ± 0.41	-0.37 [-1.28 to 0.54]		
Control	2	2.28 ± 0.16	1.43 ± 0.62	-0.85 [-1.08 to 0.91]	0.49 [-1.30 to 2.27]	
No						
Exercise	13	1.80 ± 0.20	1.80 ± 0.21	0.01 [-0.46 to 0.47]		
Control	13	1.84 ± 0.32	1.79 ± 0.59	-0.05 [-0.62 to -0.52]	0.05 [-0.64 to 0.75]	

Legend: Menstrual dysfunction was defined as i) cycles shorter than 24 days, or ii) one cycle longer than 36, or iii) average mid-luteal salivary progesterone values 0.11-0.2 ng/ml (at least two samples available).

Figure 1 – Diagram of research design based on day of menstrual cycle

Day 19-24	Recruitment and initial screening Fitness test

1st Cycle	**All participants**
Day 1	Start of Control Cycle
Day 3-6	1 Follicular Phase Urine Sample
Day 19-23	1 Luteal Phase Urine Samples 3 Day Diet Record 3 Luteal Phase Saliva Samples
Day 19-24	DEXA
Day 25	Randomization

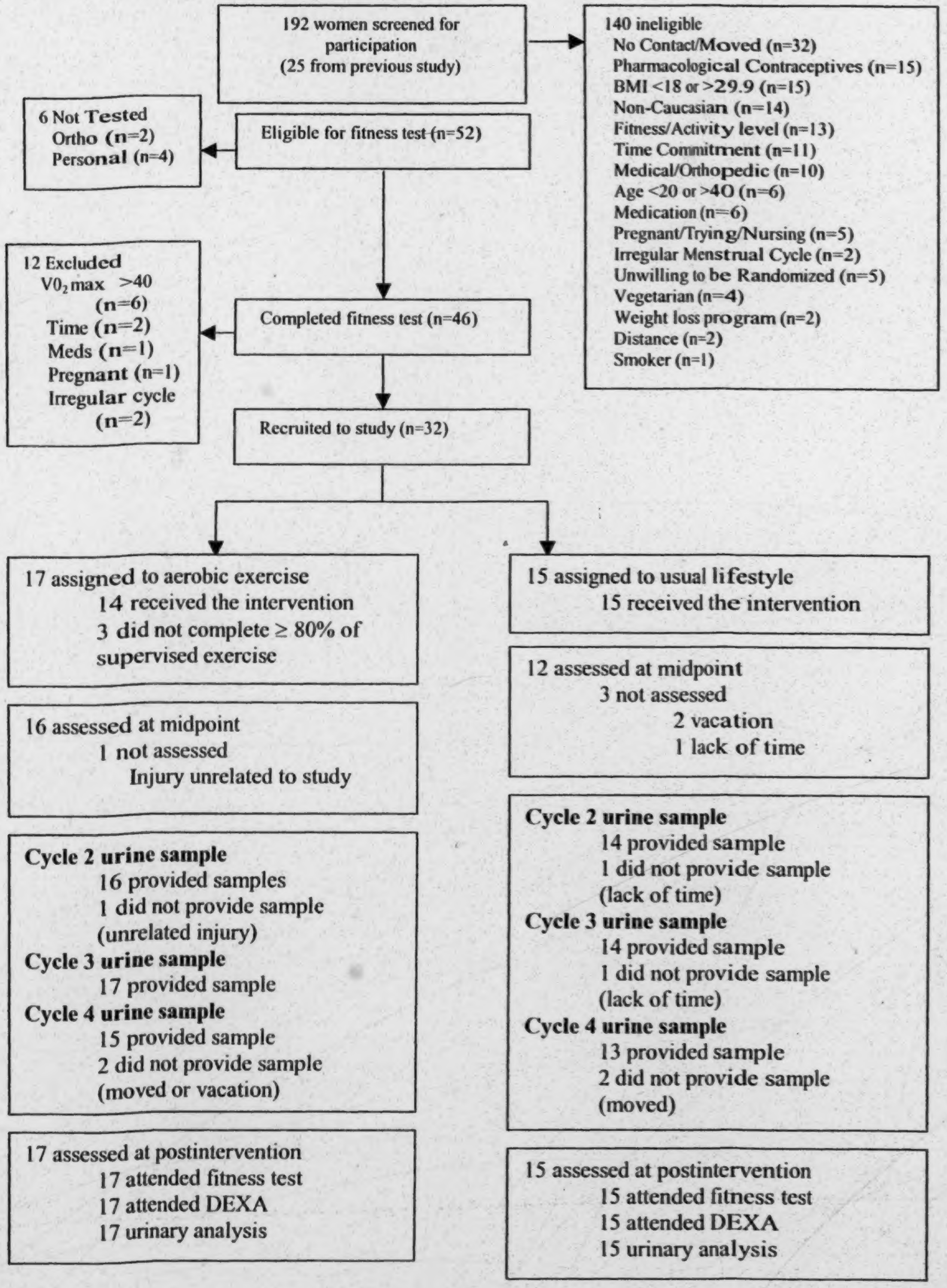
↓

2nd & 3rd Cycle	**Exercise Group and Control Group**	
Day 1	**Exercise Group Only** Start 3-4 days/week supervised activity	**Control Group** Usual lifestyle
Day 3-6	1 Follicular Phase Urine Sample	
Day 19-23	1 Luteal Phase Urine Samples	

↓

4th Cycle	**Exercise Group and Control Group**
Day 3-6	1 Follicular Phase Urine Sample
Day 19-23	1 Luteal Phase Urine Samples 3 Day Diet Record 3 Luteal Phase Saliva Samples
Day 19-24	Aerobic Fitness test DEXA

Figure 2 — Participant flow through the trial


192 women screened for participation (25 from previous study)
140 ineligible
No Contact/Moved (n=32)
Pharmacological Contraceptives (n=15)
BMI <18 or >29.9 (n=15)
Non-Caucasian (n=14)
Fitness/Activity level (n=13)
Time Commitment (n=11)
Medical/Orthopedic (n=10)
Age <20 or >40 (n=6)
Medication (n=6)
Pregnant/Trying/Nursing (n=5)
Irregular Menstrual Cycle (n=2)
Unwilling to be Randomized (n=5)
Vegetarian (n=4)
Weight loss program (n=2)
Distance (n=2)
Smoker (n=1)
Eligible for fitness test (n=52)
6 Not Tested
Ortho (n=2)
Personal (n=4)
Completed fitness test (n=46)
12 Excluded
V0₂ max >40 (n=6)
Time (n=2)
Meds (n=1)
Pregnant (n=1)
Irregular cycle (n=2)
Recruited to study (n=32)
17 assigned to aerobic exercise
14 received the intervention
3 did not complete ≥ 80% of supervised exercise
15 assigned to usual lifestyle
15 received the intervention
16 assessed at midpoint
1 not assessed
Injury unrelated to study
12 assessed at midpoint
3 not assessed
2 vacation
1 lack of time
Cycle 2 urine sample
16 provided samples
1 did not provide sample (unrelated injury)
Cycle 3 urine sample
17 provided sample
Cycle 4 urine sample
15 provided sample
2 did not provide sample (moved or vacation)
Cycle 2 urine sample
14 provided sample
1 did not provide sample (lack of time)
Cycle 3 urine sample
14 provided sample
1 did not provide sample (lack of time)
Cycle 4 urine sample
13 provided sample
2 did not provide sample (moved)
17 assessed at postintervention
17 attended fitness test
17 attended DEXA
17 urinary analysis
15 assessed at postintervention
15 attended fitness test
15 attended DEXA
15 urinary analysis

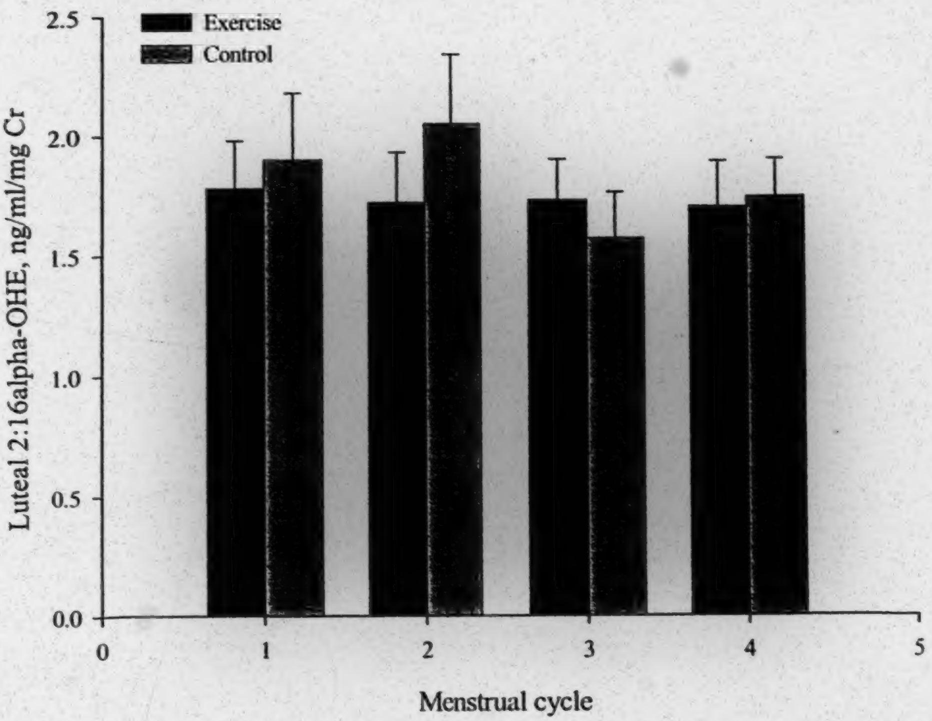


Figure 3. 2:16α-OHE1 level over the course of the intervention (N=32)

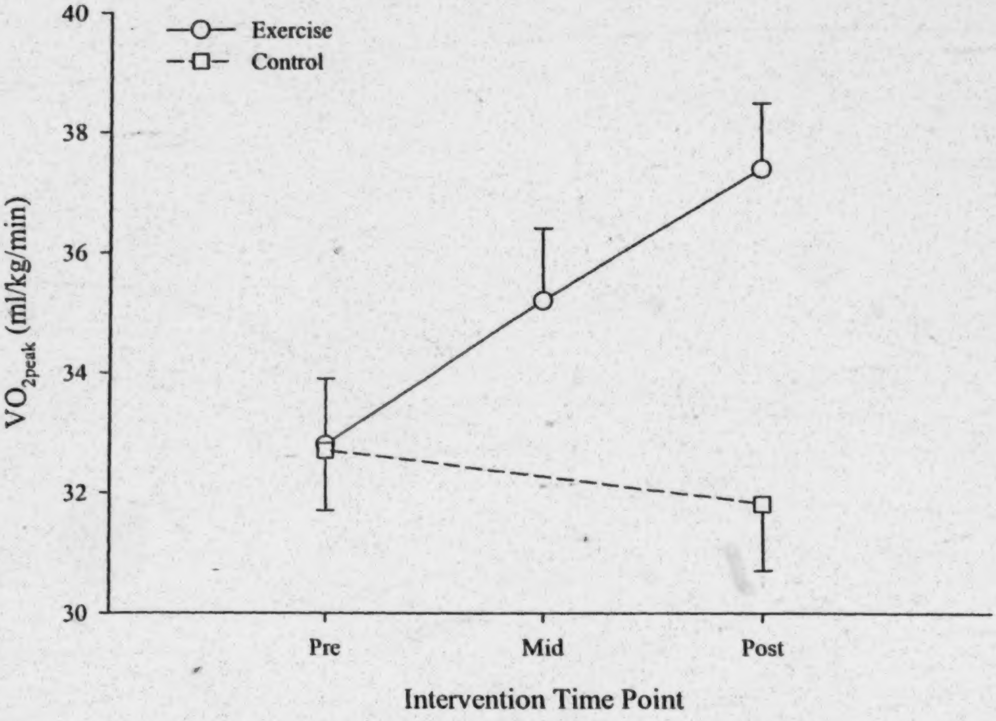


Figure 4. Aerobic fitness levels over the course of the intervention (N=32)

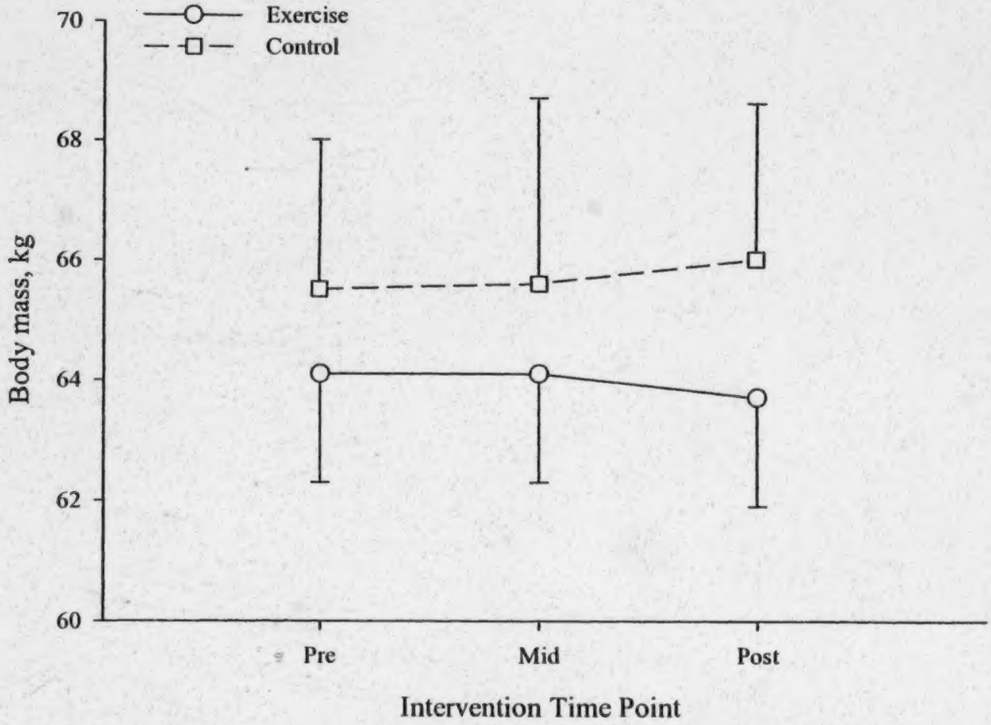


Figure 5. Body mass over the course of the intervention (N=32)

CHAPTER 5 – Overall Conclusions and Future Directions

This dissertation focused on the impact of aerobic fitness level and aerobic exercise training on estrogen metabolites, proposed biomarkers of breast cancer risk. Epidemiologic evidence suggests a 30-40% decrease risk of breast cancer in women who are physically active (1) but the biological mechanisms responsible for the association between physical activity and cancer risk reduction are still unclear. One proposed biological mechanism is alterations in estrogen metabolism, specifically the metabolites 2-hydroxyestrone (2-OHE1) and 16α-hydroxyestrone (16α-OHE1). 16α-OHE1 has been shown to be estrogenic, while 2-OHE1 is non-estrogenic (2, 3). Higher 2-OHE1 levels are suggested to have a protective effect, along with a higher 2:16α-OHE1, while higher 16α-OHE1 levels or a lower 2:16α-OHE1 are associated with an increased breast cancer risk (4, 5).

To date, the literature investigating the link between physical activity and estrogen metabolism has been limited. There are few studies, the majority of which are observational studies with small sample sizes that used self-reports of physical activity (6-10). Until recently, there was only one intervention study in this area and the physical activity component was limited to encouraging participants to be more physically active (mainly through walking) and was only one part of a larger group-focused lifestyle intervention aimed to reduce body mass (11). Since the development of this dissertation, the results of a 12-month moderate intensity aerobic exercise randomized controlled trial in post menopausal women has been published (12). In general the observational studies have found higher 2-OHE1 levels in athletes compared to controls (7), higher 2-OHE1 levels with high intensity training resulting in the development of menstrual dysfunction (7, 9, 10), and an association between 2:16α-OHE1 and self-reported physical activity (6, 8). The two intervention studies, however, have shown no effect of physical activity on 2:16α-OHE1 (11, 12).

The aim of this dissertation was to improve on the research methodology used in the previous literature to systematically examine the effects of physical activity on estrogen metabolism in premenopausal women. Study One used an objective indicator of adaptation to chronic moderate-to-vigorous physical activity, namely aerobic fitness, to compare estrogen metabolite levels in women who were highly aerobically trained (i.e. international, national, and provincial runners, cyclists and cross-country skiers, along with varsity runners, rowers and rugby players) and women who were sedentary or recreationally active. Study Two used an aerobic exercise training intervention to examine the effects of exercise training on estrogen metabolites using randomized controlled trial methodology.

Study One was a cross-sectional observational study (13). Fitness level was determined by maximal oxygen consumption (VO_{2max}) by an incremental exercise test on a cycle ergometer. High fitness was classified as a VO_{2max} ≥48 ml/kg/min, while average fitness was classified as a VO_{2max} ≤40 ml/kg/min (14). Urinary estrogen metabolite levels, namely 2-OHE1, 16α-OHE1, and 2:16α-OHE1, in both the follicular and luteal phase of the menstrual cycle were compared in 17 women with high fitness to 13 women with average fitness. Both groups were similar in age, height, body mass and body mass index (BMI). However, the high fitness group (mean=VO_{2max} 52.8 ml/kg/min) were leaner than the average fitness group (mean=VO_{2max} 35.0 ml/kg/min). No statistically significant differences in 2-OHE1, 16α-OHE1, and 2:16α-OHE1 were observed between the groups. Further analysis showed that overall, a higher BMI was associated with lower follicular 2-OHE1 ($r=-.37, p=.04$) and 2:16α-OHE1 ($r=-.40, p=.03$), and higher sum of four skinfolds was associated with a higher luteal 16α-OHE1 level ($r=.39, p=.03$) and lower luteal 2:16α-OHE1 ($r=-.41, p=.02$). These findings were consistent with a low risk of breast cancer for the leaner women.

To date this is the first published study to investigate the association between and objective measure of aerobic fitness and estrogen metabolites in premenopausal women. No association was found, which was in contrast to three previous observational studies (7, 9, 10) that found higher 2-OHE1 levels in athletic women, which were comparable to our high fitness group. However, in the previous studies the higher 2-OHE1 levels were restricted to the athletes who developed menstrual dysfunction with higher intensity training, while all the participants in Study One had regular menstrual cycles. The observed association between body composition and estrogen metabolites was consistent with the previous literature in which leanness was associated with 2-OHE1 levels (10).

Explanations for the lack of difference observed in estrogen metabolites between the high fitness and average fitness group include the use of inappropriate cutpoints for aerobic fitness to observe an association, an alternate pathway for the association between physical activity and estrogen metabolites, or sampling procedures that failed to capture possible variability across the menstrual cycle.

Study One had several strengths including an objective measure of aerobic fitness (a proxy measure of chronic moderate-to-vigorous physical activity), inclusion of only women with regular menstrual cycles (i.e. to avoid the impact of menstrual dysfunction), and inclusion of women who were reproductively mature (i.e. to avoid previous comparisons of younger athletes to older controls). However, the study also had a relatively small sample size, only measured estrogen metabolites during one menstrual cycle and assessment of body composition was limited to BMI and sum of skin folds, which are only indicators of subcutaneous fat rather than direct measures.

Study One found a non-significant increase in luteal 2:16α-OHE1 in women with high levels of aerobic fitness and indicated that body composition was associated with levels of 2-OHE1 and 16α-OHE1. Based on these findings, Study Two investigated the effect of an exercise intervention designed to improve aerobic fitness on estrogen metabolism using randomized controlled trial methodology. This type of intervention directly examined the effects of an objectively quantified exercise training program on estrogen metabolites compared to a control group over several menstrual cycles, thereby improving on some of the limitations noted in Study One. These improvements included measurement of estrogen metabolites over several menstrual cycles compared to a single menstrual cycle, producing a causal change in aerobic fitness from below average to average or from average to above average rather than the use of pre-selected extreme fitness groups, and improved measures of body composition with the use of dual-energy x-ray absorptiometry (DEXA).

Study Two involved a 12-week supervised aerobic exercise training program in premenopausal women. Thirty two women were recruited to the study and completed baseline testing, which included a maximal aerobic fitness test on a cycle ergometer (VO_{2max}) and assessment of body composition (DEXA) prior to being randomized. At baseline, there were no differences in aerobic fitness, body composition, reproductive factors or estrogen metabolites levels between the 17 participants assigned to the exercise group and the 15 participants assigned to the control group. Overall adherence to the exercise intervention was 91% and there was no loss to follow up. Despite a significant (14%) increase in aerobic fitness in the exercise group, no significant difference in luteal 2-OHE1, 16α-OHE1 or 2:16α-OHE1 was observed between the groups, moreover change in aerobic fitness was not associated with a change in estrogen metabolites. However, an increase in lean body mass was associated with an increase in 2:16α-OHE1 (r=.43, p=.015). In addition, at baseline an inverse association between 2:16α-OHE1 and percent body fat (r=-.40, p=.044) was seen for all participants, along with a positive association between abdominal fat and 16α-OHE1 (r=.43, p=.013).

To date, there are no published studies examining the effects of an aerobic exercise training intervention on the urinary excretion of estrogen metabolites 2-OHE1 and 16α-OHE1 or their ratio, 2:16α-OHE1, in premenopausal women. Overall, an increase in aerobic fitness, induced by a supervised exercise intervention, did not alter estrogen metabolite levels. This finding is consistent with the only two previous studies that have examined the effects of a physical activity intervention on levels of 2-OHE1, 16α-OHE1, or their ratio, 2:16α-OHE1(11, 12) but is inconsistent with several observational studies that suggest physical activity is associated with estrogen metabolite levels (7-10). However, changes in body composition were associated with changes in estrogen metabolite levels. These findings are consistent with the recently published randomized controlled trial of a 12-month moderate physical activity intervention in postmenopausal women, which reported a positive association between and increase in lean mass and change in 2:16α-OHE1, and also the observational studies which have reported associations between physical activity and body composition in some (6, 7, 10, 15-17), but not all studies (18).

The overall findings of Study One and Study Two suggest that level of aerobic fitness as assessed by VO_{2max} and aerobic exercise training may not directly impact estrogen metabolism. No difference in 2-OHE1, 16α-OHE1 or 2:16α-OHE1 was observed in a comparison of women with high aerobic fitness and women with average aerobic fitness. In addition, changing from below average (mean=VO_2peak 32.8 ml/kg/min) to slightly above average fitness (mean=VO_2peak 37.4 ml/kg/min) aerobic fitness, did not alter 2-OHE1, 16α-OHE1 or 2:16α-OHE1. This suggests that if physical activity does alter estrogen metabolites it is not mediated by changes in aerobic fitness of this magnitude. This finding is consistent with the epidemiological evidence linking physical activity and reduced breast cancer risk. The observed associations have been based on higher levels of self-reported physical activity, not high levels of aerobic fitness (1, 19).

Weight maintenance, weight loss and reduction in adiposity have been proposed to be one mechanism through which physical activity reduces risk of breast cancer in postmenopausal women (20). The associations observed in both of the studies in this dissertation suggest that body composition, particularly higher body fat, is associated with a higher 16α-OHE1, lower 2-OHE1 and lower 2:16α-OHE1, a less favourable pattern of estrogen metabolites that has been linked to higher breast cancer risk. This is consistent with the literature which has reported lower 2-OHE1 levels in obese individuals compared to those of normal body weight (17), and higher 2-hydroxylation in anorexic women compared to normal weight and obese women (16). This suggests that if physical activity is to play a role in possibly altering estrogen metabolites, it may be mediated through fat loss. Exercise interventions should focus on weight maintenance throughout life, weight loss in overweight individuals and lowering overall adiposity. These types of outcomes could be achieved by physical activity that may not lead to large changes in aerobic fitness.

In Study Two, an increase in lean body mass was associated with a more favourable 2:16α-OHE1, similar to the association noted by Atkinson et al. (12). To date, a mechanism for alterations in 2:16α-OHE1 with a gain in lean mass has not been suggested and the overall impact of lean body mass on estrogen metabolism has not been reported. These findings suggest that a resistance exercise intervention to increase lean body mass may alter estrogen metabolism.

Based on the current literature, future research aimed at altering estrogen metabolism should focus on altering body composition. Interventions should aim to induce a negative energy balance, either through altering dietary intake, physical activity or a combination of the two. In addition, studies to further investigate the recently observed associations between changes in lean body mass and alterations in estrogen metabolism are warranted. Furthermore, the modest variability of estrogen metabolite levels within individuals and wide variability across individuals warrants further study. There is limited literature on expected normal values and expected change over short and longer time frames. Additional research that provides this type of information would be helpful in understanding the results of both observational studies and intervention trials. Finally, a greater understanding of the mechanism responsible for shifts in estrogen metabolism is needed. This involves the examination of associated enzymatic activity and polymorphisms related to estrogen metabolism, and their interaction with lifestyle factors. This would provide not only better mechanistic knowledge to guide interventions but also may identify individuals in which interventions may be more effective.

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Appendix 1 - Avenue of recruitment for screening and randomization into the trial

	Screened	Randomized
Faculty of Nursing	51 (24%)	12 (38%)
Graduate Students Association	37 (19%)	8 (25%)
Edmonton Examiner Newspaper	34 (18%)	4 (13%)
Undergraduate Health Education classes	16 (8%)	3 (9%)
Study One	13 (7%)	3 (9%)
Other studies in Faculty	12 (6%)	0
Edmonton AM CBC Radio	8 (4%)	1 (3%)
Friend/Word of Mouth	6 (3%)	1 (3%)
Other	3 (2%)	0
Unknown	12 (6%)	0
TOTAL	192	32

Presented as total number of women who were screened and percentage of the total.

Appendix 2 – Telephone screening tool

HORMONE STUDY

Eligibility Criteria Script

Thanks for your interest in the study.

This study will look at the effect of physical activity on sex steroid hormones (such as estrogen) and estrogen metabolite levels. Large population studies have shown that women who are more active may have a lower risk of breast cancer, but the reasons for this are not known. One possible reason for the lower risk seen with physical activity, is a possible shift in estrogen metabolism pattern. However, the relationship between activity level and estrogen metabolism pattern has not been fully investigated. This research is very preliminary and will in no way quantify your cancer risk.

Before I explain the protocol of the study, I would like to ask you a few questions to see if you will be eligible to participate. All the questions have to do with things that may affect hormone function. Is if ok to ask you these questions?

1. How old are you? ____________

2. Is your menstrual cycle, on average, between 24-36 days long? ______________
[From the start of one menstrual period to the start of another, how many days is it usually].

3. Have you had at least 10 menstrual cycles in the past year? Yes ☐ No ☐

4. Are you a smoker? Yes ☐ No ☐

If not, have you been in the past year? Yes ☐ No ☐
(eligible if no cigarette use for at least one year)

5. Are you currently on pharmacological contraceptives (oral contraceptive pills, injection, or patch)? Yes ☐ No ☐

If not, have you been in the past 6 months? Yes ☐ No ☐
Type ______________________________ Stop date _________________________

6. Are you Caucasian? Yes ☐ No ☐ ____________________________________

7. Do you feel that you will be able to travel to the University of Alberta for the initial exercise test, and 12 week exercise program 3 times per week?
Yes ☐ No ☐ ____

6. Are you a vegetarian? Yes ☐ No ☐

7. What is your height ___________ and weight _____________
See BMI sheet (eligible if between 18-29.9)

8. Do you have any significant medical conditions? ____________________________

Have you been told that you have an endocrine condition?
☐ Diabetes ☐ Thyroid

Are you currently taking any medications?
___________________________________ For ___________________________

___________________________________ For ___________________________

___________________________________ For ___________________________

9. Do you have any orthopedic/muscular or joint injuries that would prevent you from doing a maximal exercise test on a stationary bike?
Yes ☐ No ☐
____

10. Godin Leisure Time Physical Activity Questionnaire.

Considering the past 7 days, how many times did you do the following kinds of exercise for more than 20 minutes during your free time.

1. STRENUOUS EXERCISE
-heart beats rapidly, sweating, breathing much harder than normal
-i.e. running, vigorous aerobics classes, cross country skiing, vigorous swimming, vigorous bicycling

Times per week: ______________

Average Duration: _______________________

Type of activity: ____

2. MODERATE EXERCISE
-not exhausting, light perspiration, breathing slightly harder than normal
-i.e. fast walking, tennis, easy bicycling, easy swimming, dancing

Times per week: ____________________

Average Duration: _____________________

Type of activity: __________________________

3. MILD EXERCISE
-minimal effort, no perspiration
-i.e. easy walking, yoga, golf, bowling

Times per week: ___________________

Average Duration: ____________________

Type of activity: ____________________

4. Additional screening question: Is this typical of your activity in:

- the last 3 months? Y N
- the last 6 months? Y N
- the last year? Y N

Contact Information: Name ____
Phone number h. ____________________ w./c. ____________________

Time to call ____

Appendix 3 – Rationale for eligibility criteria

1) Female

Breast cancer occurs in men but is very rare. An estimated 150 Canadian men will be diagnosed with breast cancer in 2005, and 45 will die from it, compared to an estimated 21,600 Canadian women who will develop breast cancer and 5,300 who will die from it (1). Given the low numbers of men diagnosed with the disease and the possibility that the etiology of the disease may be different for men and women, the study was restricted to women.

2) Caucasian

Estrogen metabolism ratios have been noted to vary with ethnicity (2-5). The present study focused on one ethnicity to eliminate a possible baseline difference in estrogen metabolism in participants and it is possible that factors which may alter estrogen metabolism may vary by ethnicity.

3) Sedentary or recreationally active

The operational definition of sedentary was not engaging in vigorous intensity exercise, for 20 minutes or more, three or more times per week in the past six months, and no history of significant aerobic training in the past year. Recruiting sedentary participants allowed for the effects of an exercise intervention to be more apparent that using an already active cohort.

4) 20 -35 years of age

Women in this age range are more likely to have regular menstrual cycles. At age 20 years, participants are farther from onset of menses and are more likely to have established regular menstrual cycles, and at age 35 years are less like to be undergoing significant peri-menopausal changes to menstrual function (6).

5) Self reported regular menstrual cycles

This was defined as a menstrual cycle intervals of 24-36 days and at least 10 menstrual cycles in the past year to ensure participants did not have an underlying menstrual dysfunction or recent history of periods of oligomennorrhea, and were not pregnant (7).

6) Body mass index (BMI) 18 – 29.9

A body mass index above or below this range may be associated with menstrual dysfunction (8). The inclusion of both normal body mass and overweight women improved the generalizability of the findings to the general population. In 1996/97, about half of Canadian adults were in the acceptable body mass range; 34% were overweight (9).

7) *Not using pharmacological contraceptives (currently, or in the past 6 months)*

Pharmacological contraceptives could influence the results of the hormonal outcome measures beyond the impact of physical activity alone (7, 10)

8) *Non-smoker (for previous smokers, no cigarette use for at least one year)*

Smoking has been shown to alter estrogen profiles (7) and estrogen metabolites (11, 12).

9) Not vegetarian

Dietary intake has been shown to alter estrogen metabolite levels, particularly soy intake and certain vegetables (i.e. *Brassica* vegetables) (13).

10) No reported endocrine abnormality (i.e. diabetes or thyroid dysfunction)

This could alter normal menstrual function and limit ability to participate in exercise (7).

11) No use of medications that may interfere with hormonal status

To limit the additional effects of factors that have been shown to alter normal menstrual function (7).

12) Does not have any contraindications to maximal exercise test

As determined by the Physical Activity Readiness Questionnaire (PAR-Q) (14) screening questionnaire. This would have prevented a participant from undertaking an exercise intervention.

13) Geographically accessible

Living within one-hour drive of the University of Alberta to facilitate ease of participation in a supervised exercise program and biological sample collection.

14) Plans to be away for more than seven consecutive days during the 12-week intervention

This would have limited the delivery of the exercise intervention.

15) Willing to be randomized

The importance of maintaining group assignment and the fundamental principles behind randomized controlled trial methodology were explained to all participants and only those who were willing to be randomized were recruited to the study.

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Appendix 4 – Informed Consent Letter

Title of Project: A randomized controlled trial of aerobic activity in premenopausal women: The effect on estrogen metabolism.

Principal Investigator: Kristin Campbell, B.Sc. PT., M.Sc.
PhD student
Faculty of Physical Education and Recreation
University of Alberta
492-2829

Co-Investigators:
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University of Alberta (780) 492-1023
Christine Friedenreich, PhD
Scientist, Alberta Cancer Board, Calgary, Alberta (430) 944-1841

PARTICIPANT INFORMATION LETTER

This consent form is part of the process of informed consent. It is designed to give you an idea of what this research study is about and what you will be asked to do if you choose to take part in the study. If you would like to know more about something mentioned in this form, or have any questions regarding this research study, please be sure to ask the Principal Investigator (Kristin Campbell).

What is this study about?

This study will look at how physical activity may change how estrogen is processed by your body. Large population studies have shown that an increase in activity level may lower risk of getting breast cancer. However, the reason for this has not been identified. One possible explanation is that physical activity may change how estrogen is processed by the body.

What is involved in participation?

To participate in this study, you were asked for some demographic information and about your usual physical activity level over the phone. This was to determine if you meet the inclusion criteria for the study. The second part of screening is an exercise test at the University of Alberta campus. The exercise test is a progressive, incremental exercise test to exhaustion (until you say that you cannot continue). The exercise intensity is quite light at the beginning of the test, and becomes more difficult every minute or two. Usually, the actual test lasts for about 10-12 minutes, with an additional 5-10 minutes of warm-up and cool-down exercise before and afterwards. During the test, the air you exhale from your mouth will be collected using a mouthpiece. Heart rate will be monitored continuously with a heart rate monitor and blood pressure will be measured at the mid-point of the test. Your height, weight and body composition (waist and hip circumference, along with five skin fold sites) will be measured.

Following the aerobic testing, you may be ineligible to participate in this study as we are looking for a specific range of scores. If you are eligible for the study, you will be *randomly assigned* to either an exercise group or a control group. Randomization will be done using numbered, sealed envelopes, and you will have an equal chance of being in either group. The actual "experiment" will involve your next **four** consecutive menstrual cycles. The first menstrual cycle is a baseline cycle, and the intervention will then take place over the following three menstrual cycles.

For **all** menstrual cycles, you will be asked to complete the following:

1) Menstrual cycle log
    - *When:* Starting on the first day of menstruation (day 1).
    - *Where:* At home
    - *What:* To provide us with an idea of your usual menstrual cycle length
    - *How:* Record the start date of each cycle and characteristics (i.e. days of menstruation, amount of flow) of the cycle.
    - *Time:* 10 minutes per cycle
2) Urine sample
    - *When:* Early in your cycle (day 4, 5 or 6 depending on your availability), and near the end of your cycles (day 20, 21 or 22; depending on your availability).
    - *Where*: Collect at home and store in your home freezer.
    - *What*: To measure estrogen metabolites and estrogen levels
    - *How:* Fast for 10 hours overnight and collect a urine sample (approximately ¼ cup) the next morning in the provided container. You will then be asked to deliver the urine sample to the University of Alberta and have your body weight and skin folds recorded. You will not get to see these measurements during the intervention.
    - *Time:* 30 minutes per cycle

3) Saliva samples

- *When:* Every other morning for six days (approximately day 19, 21, 23; but this will be individualized to you)
- *Where:* Collect at home and bring to the University of Alberta
- *What:* To measure progesterone levels
- *How:* Fast for 10 hours overnight and collect a saliva sample (approximately 1 teaspoon) the next morning in the provided container and freeze each in your home freezer. You will be asked to pack the saliva samples in ice and bring them to the University of Alberta the next time you are delivering a urine sample.
- *Time:* One hour per cycle
-

At the **start** of the study and at the **end**. You will be asked to complete:

1) Three day diet record

- *When:* Near the end of your first and last menstrual cycle of the study (day 18-24)
- *Where:* At home, work, school.
- *What:* Provides us with an idea of your usual diet
- *How:* Record what you eat for three consecutive days, including brand names and sizes (teaspoons, table spoons, centimetres, millilitres, ounces, etc.)
- *Time:* A half hour daily for three days

2) Exercise Test

- *When:* Near the end of your last menstrual cycle of the study (day 18-24)
- *Where:* Exercise Testing Room (Cross Cancer Institute)
- *What:* A measure of your aerobic fitness level
- *How:* Progressive cycle ergometer test to exhaustion that is exactly the same as the initial fitness test.
- *Time:* One hour

3) DEXA (dual-energy x-ray absorptiometry) scan

- *When:* Near the end of your first and last menstrual cycle of the study (day 18-24)
- *Where:* Human Nutrition Research Centre (University of Alberta)
- *What:* Measures body composition (total muscle, bone and fat mass) using very low dose radiation [see attached DEXA Information Sheet].
- *How:* Lie on scanner and scanning arm passes over you
- *Time:* 20 minutes
- *Note:* All participants will be asked to undergo a pregnancy test prior to the scan (to prevent radiation exposure to unborn fetus)

4) Fasted blood sample

- *When:* During the first half of your first menstrual cycle and the first half of the menstrual cycle after you finish the intervention (day 3-8)
- *Where:* College Plaza
- *What:* A needle to take blood from the vein close to your elbow (about 2 tablespoons).
- *How:* At the blood collection lab by a trained technician.
- *Time:* 20 minutes

The total number of samples: eight urine samples, twelve saliva samples, two blood samples and one cheek cell sample over four months. In addition, you will be asked to complete two exercise tests, two diet records, and two DEXA scans.

What does each group do?

This study is a randomized controlled trial. You will be followed for one menstrual cycle (the control cycle) and at the end of that menstrual cycle, you will have an *equal* chance of being randomly assigned to: 1) the exercise group, or 2) the control group for your next three menstrual cycles.

The ***exercise group*** will be given a customized and supervised aerobic exercise program that will last for three menstrual cycles (approximately 90 days). The aerobic exercise will take place in the Behavioural Medicine Fitness Centre (University of Alberta Campus) three days per week. The sessions will run anytime between 7 am-7 pm Monday to Friday and 9 am-1 pm on Saturday. The exercise program will consist of 30-45 minutes of *moderate to vigorous* aerobic exercise per session. Examples of moderate intensity activity are things like brisk walking or biking that causes you to increase your breathing rate. Examples of vigorous intensity activity are things like jogging, and fast cycling that cause you to be more out of breath. Each exercise training session will take about 45-60 minutes (including warm-up and cool-down). You will be asked to start with three supervised sessions per week *using the bicycles* at the Behavioural Medicine Fitness Centre. After 4 weeks, you will be asked to add an additional session (for a total of 4 sessions per week). The additional sessions can be completed on any of the aerobic equipment at the Behavioural Medicine Fitness Centre.

The ***control group*** will be asked to maintain their normal lifestyle. This means continuing with usual physical activity and eating patterns, and not starting a structured exercise program or weight loss program. At the end of the study, individuals in the control group will receive a customized aerobic exercise program and access to supervision at the Behavioural Medicine Fitness Centre (University of Alberta Campus) three days per week for one month.

What is the time commitment?

We estimate that the total time commitment of testing, including urine saliva, and blood sample collection, for all participants will not exceed 10 hours. Tests and sample delivery will be scheduled at mutually convenient times, including early morning, during the "normal" workday, or during the early evening. In addition, the exercise group will be asked to perform approximately 2.5-4.5 hours per week of supervised physical activity at the Behavioural Medicine Fitness Centre. The time commitment is 4 hours per week for three menstrual cycles, which is a total of approximately 40 additional hours for the exercise group. All parking costs will be covered by the study.

Are there any risks involved?

The ***aerobic fitness test*** requires maximal effort in order to go to exhaustion. With this type of exercise there may be some risk. During and after the tests it is possible to experience symptoms such as abnormal blood pressure, fainting, light-headedness, muscle cramps or strain, nausea, and in very rare cases (0.5 per 10,000 in testing facilities such as exercise laboratories, hospitals and physicians' offices) heart rhythm disturbances or heart attack. While serious risk to healthy participants is highly unlikely, they must be acknowledged, and participants willingly assume the risks associated with very hard exercise. The exercise test will be administered by appropriately trained personnel. Personnel are trained to handle identifiable risks and emergencies, and have certification in CPR.

The x-ray dose associated with the ***total body composition measurements*** using DEXA (dual-energy x-ray absorptiometry) is very low and safe for repeated measurements. With the **exception** of pregnant women, there are no known risks associated with a DEXA. The potential risks associated with radiation exposure to an unborn fetus are not known, and therefore we ask that you undergo a pregnancy test to verify that you are not pregnant.

During the occasions on which you have ***blood drawn*** you may have a slight degree of discomfort. The blood sample will result in a puncture of your skin, which may lead to a bruise or infection if not treated properly. These risks will be minimized, as sampling will be performed by a trained professional.

Given the ***instrumentation used to collect the information*** in this study, i.e. fitness testing and body composition scans, there are risks associated with participation that revolve around the disclosure of personal or sensitive information. This may make some participants uncomfortable. If requested, referral to a counsellor or source of appropriate information or professional advice will be provided.

Are there any benefits to participation?

If following baseline testing you are ineligible for the study, you will be provided with your maximal aerobic exercise test results and interpretation. If you are eligible for the study, you will be provided with a summary sheet of results for your fitness test, dietary analysis, and body composition at the end of the study. Also all eligible participants will receive an individualized exercise prescription and supervision (either during the study or following the study for the control group). While the additional information gathered from this study will not benefit you personally, it will help to add to our knowledge of breast cancer risk factors and how physical activity may alter these possible risk factors.

How confidential are my results?

To ensure anonymity, personal information will be coded and stored in a secure location (e.g., a locked file cabinet and secure computer) that is accessible only to the researchers. If you withdraw from the study, your information will be removed from the results on your request. The coded results will be used for scientific research only. You will not be identified in any presentation or publication. Normally, information is retained for a period of five years post publication after which it will be destroyed.

Can I withdraw from the study?

You are free to withdraw at any time without explanation and without any consequences. You may withdraw by contacting the principal investigator in person, by phone, or by email. If you do choose to withdraw, your information will be removed from the study at your request.

What if I have questions or concerns about this research?

If you have any questions about your participation in the study, please contact one of the researchers. If you have other concerns about this research, you may contact Dr. Brian Maraj, Chair, Faculty of Physical Education and Recreation Research Ethics Committee at the University of Alberta at (780) 492-5910. His office has no direct affiliation with this study.

____

Kristin Campbell, Principal Investigator

August 2004

CONSENT FORM

Title of Project: A randomized controlled trial of aerobic activity in premenopausal women: The effect on estrogen metabolism.

Principal Investigator(s): Kristin L. Campbell (780)492-2829

Co-Investigator(s):	Include affiliation(s) and phone number(s):
Kerry Courneya	Professor, PER, University of Alberta 492-1031
Kim Westerlind	Scientist, AMC Cancer Centre, Denver, CO (303)
239-3472	
Vicki Harber	Associate Professor, PER, University of Alberta
492-1023	
Christine Friedenreich	Scientist, Alberta Cancer Board, Calgary, AB (403)
944-1841	

Do you understand that you have been asked to be in a research study?	Yes	No
Have you read and received a copy of the attached Information Sheet	Yes	No
Do you understand the benefits and risks involved in taking part in this research study?	Yes	No
Have you had an opportunity to ask questions and discuss this study?	Yes	No
Do you understand that you are free to refuse to participate, or to withdraw from the study at any time, without consequence, and that your information will be withdrawn at your request?	Yes	No
Has the issue of confidentiality been explained to you? Do you understand who will have access to your information?	Yes	No

This study was explained to me by: ________________________________

I agree to take part in this study:

Signature of Research Participant ____________________

Date ____________________

Witness ____________________

Printed Name ____________________

Printed Name ____________________

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature of Investigator or Designee ____________________

Date ____________________

Appendix 5 - Participant's Responsibilities

Participant's duties:

1. To complete all questionnaires and return them to the Study Coordinator in a timely manner at baseline, mid-point, and completion of the study.
2. To undergo a maximal aerobic fitness test at baseline, and completion of the study.
3. To complete a four fasted blood and two urine samples for each menstrual cycle in the study.
4. To be randomized to an exercise or non-exercise control.
5. For the exercise group: to participant in an 8-week aerobic training program, three to four times a week.
6. For the control group: to continue their present lifestyle for the duration of the study.

I, ______________________________ (print name) have read the participant's responsibilities and I understand what is expected of me and agree to participate in the study.

______________________________ ______________

Participant's signature Date

______________________________ ______________

Witness' signature Date

Appendix 6 – General Demographic form

Faculty of Physical Education and Recreation

PHYSICAL ACTIVITY AND ESTROGEN STUDY

Demographic Questionnaire

Study ID: ________________

A. DEMOGRAPHIC INFORMATION

A1. **When were you born?** ☐☐ / ☐☐ / ☐☐☐☐
Day Month Year

A2. **What is your current marital status?**

○ Married
○ Common-law
○ Separated
○ Divorced
○ Widowed
○ Never Married
○ Other
____________ specify

A3. **What is the highest level of school that you have completed?**

○ Did not complete grade school
○ Grade school
○ High school
○ College or trade school
○ University undergraduate degree
○ University graduate degree

A4. **To which ethnic or cultural groups did you, or most of your ancestors belong, on first coming to North America?**

○ British
○ Western European
○ Eastern European
○ French
○ Northern European
○ Southern European
○ Aboriginal
○ East and Southeast Asian
○ Southern Asian
○ Western Asian
○ Pacific Islands
○ Arab
○ Latin, Central and South American
○ Caribbean
○ African
○ Other ________________ (specify)

. MENSTRUAL HISTORY

B1. **How old were you when your menstrual periods started?**

☐☐ (years)

C. REPRODUCTIVE HISTORY

C1. **Have you ever been pregnant?**

○ Yes **If no, go to section D**
○ No

C2. **How many pregnancies have you had?**

☐☐ **Total number of pregnancies including miscarriages, abortions, still births and live births**

C3. **How many live births have you had?**

☐☐ **Total number of pregnancies that resulted in live births**

C4. **How old were you when your first child was born?**

☐☐ **years**

D. MEDICAL HISTORY

D1. **Have you ever been diagnosed with any of the following conditions?**

High cholesterol or triglycerides	○ Yes	○ No
Heart attack (myocardial infarction)	○ Yes	○ No
Cardiac chest pains (angina pectoris)	○ Yes	○ No
Stroke	○ Yes	○ No
Arthritis (rheumatoid arthritis or osteoarthritis)	○ Yes	○ No
Osteoporosis	○ Yes	○ No
Blood clots in the veins of your legs or pelvis	○ Yes	○ No
Blood clot in your lungs	○ Yes	○ No
Thyroid problems	○ Yes	○ No
Any other medical conditions	○ Yes	○ No

If yes, what conditions?

D2. **Have any of your mother, daughters or sisters had breast cancer? Do not include any stepsisters, half sisters or adopted sisters.**

○ Yes ○ No

Appendix 7 – Exercise Intervention Sheets

HORMONE II

Participant ID
Week 1
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX [illegible]

Session:	Date:	Type	Time	Intensity	Equipment
1		Tempo	30		Bike
2		Tempo	35		Bike
3		Tempo	40		Bike

1

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

2

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

3

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

HORMONE II

Participant ID
Week 2
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX [illegible]

Session:	Date:	Type	Time	Intensity	Equipment
1		Tempo	35		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

2		Tempo	30		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

3		Tempo	30		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

HORMONE II

Participant ID:
Week: 3
DATE:

Name:
Home:
Work:
Email:

TEMPO: THRESHOLD: MAX:

Session:	Date:	Type	Time	Intensity	Equipment	Time	Av. HR	Wattage	RPE	Equipment
1		Tempo	10		Bike					
2		Tempo	16		Bike					
3		Tempo	[illegible]		Bik					

Comments (session 1): HR = / RPE =

Comments (session 2): HR = / RPE =

Comments (session 3): HR = / RPE =

HORMONE II

Participant ID
Week 4
DATE:

Name:
Home:
Work:
Email:

[illegible]

Session:	Date:	Type	Time	Intensity	Equipment	Time	Av. HR	Wattage	RPE	Equipment
1		Tempo		15	Bike					

Comments:
HR =
RPE =

		Type	Time	Intensity	Equipment	Time	Av. HR	Wattage	RPE	Equipment
2		Tempo		15	Bike					

Comments:
HR =
RPE =

		Type	Time	Intensity	Equipment	Time	Av. HR	Wattage	RPE	Equipment
3		Tempo		15	Bike					

Comments:
HR =
RPE =

HORMONE II

Participant ID
Week 5
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX

Session:	Date:	Type	Time	Intensity	Equipment
1		[illegible]	[illegible]		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: 10 min warm up; 2 x 10 min at threshold; 10 min rest; 10 min cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
2		Tempo	[illegible]		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
3		VO2 m	[illegible]		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: 10 min warm up (work up close to max wattage for about 30 sec); 2 x 10 30 sec ON 30 sec OFF; 10 min rest
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
4		Tempo	[illegible]		Anything

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

HORMONE II

Participant ID:
Week: 6
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX

Session:	Date:	Type	Time	Intensity	Equipment
1		[illegible]	20		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: 10 min warm up; 2 x 10 min at threshold; 10 min rest; 10 min cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
2		Tempo	10		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
3		VO2 max	[illegible]		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: 10 min warm up (work up close to max wattage for about 30 sec); 2 x 12 30 sec ON 30 sec OFF; 10 min rest
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
4		Tempo	[illegible]		Anything

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

HORMONE II

Participant ID
Week 7
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX [illegible]

Session:	Date:	Type	Time	Intensity	Equipment
1		[illegible]			Bike

Comments:
HR =
RPE =

2		[illegible]		[illegible]	Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

3		[illegible]		[illegible]	Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
Warm up; 20 x 30 sec ON; 30 sec OFF; cool down
HR = RPE =

4		[illegible]		[illegible]	Anything

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

HORMONE II

Participant ID
Week 8
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX

Session:	Date:	Type	Time	Intensity	Equipment
1		Threshold	20		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: 10 min warm up; 2 x 10 min at threshold; 10 min rest; 10 min cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
2		Tempo	45		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
3		VO2 max	20		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: Warm up; 20 x 30 sec ON; 30 sec OFF; cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
4		Tempo	45		Anything

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

HORMONE II

Participant ID
Week 9
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX [illegible]

Session:	Date:	Type	Time	Intensity	Equipment
1		VO2 Max	20		Bike

	1	2	3	4	RPE
Peak HR					
Wattage					

Comments: 10 min warm up (get close to max wattage, then back down); 2 min ON; 3 min OFF x 4; 10 min cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
2		Recovery	45		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
3		Threshold	20		Bike

	Time	Av. HR	Wattage	RPE	Equipment
1					
2					

Comments: 10 min warm up; 2 x 10 min @ Threshold; 10 min rest; 10 min cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
4		Tempo Grinds	40		Anything

Time	Av. HR	Wattage	RPE	Equipment

Comments: 8 mins at tempo and 2 mins grinds (lower cadence (50-60 rpm), increase resistance to approx. threshold W)x 4
HR =
RPE =

HORMONE II

Participant ID
Week 10
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX

Session:	Date:	Type	Time	Intensity	Equipment		1	2	3	4	RPE
1		VO2 max	20		Bike	Peak HR					
						Wattage					

Comments: 10 min warm up (get close to max wattage, then back down); 2 min ON; 3 min OFF x 4; 10 min cool down
HR =
RPE =

2		Recovery	15		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments: HR =
RPE =

3		Threshold	20		Bike

	Time	Av. HR	Wattage	RPE	Equipment
1					
2					

Comments: 10 min warm up; 2 x 10 min @ Threshold; 10 min rest; 10 min cool down
HR =
RPE =

4		Tempo Grinds	40		Anything

Time	Av. HR	Wattage	RPE	Equipment

Comments: 8 mins at tempo and 2 mins grinds (lower cadence (50-60 rpm), increase resistance to approx. threshold W)x 4
HR =
RPE =

HORMONE II

Participant ID
Week 11
DATE:

Name:
Home:
Work:
Email:

TEMPO	THRESHOLD	MAX

Session:	Date:	Type	Time	Intensity	Equipment
1		[illegible]	20		Bike

	1	2	3	4	RPE
Peak HR					
Wattage					

Comments: 10 min warm up (get close to max wattage, then back down) 2 min ON; 3 min OFF x 4; 10 min cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
2		[illegible]	45		Bike

Time	Av. HR	Wattage	RPE	Equipment

Comments:
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
3		[illegible]	[illegible]		Bike

	Time	Av. HR	Wattage	RPE	Equipment
1					
2					

Comments: 10 min warm up; 2 x 10 min @ Threshold; 10 min rest; 10 min cool down
HR =
RPE =

Session:	Date:	Type	Time	Intensity	Equipment
4		Tempo Grinds	[illegible]		Anything

Time	Av. HR	Wattage	RPE	Equipment

Comments: 8 mins at tempo and 2 mins grinds (lower cadence (50-60 rpm), increase resistance to approx. threshold W)x 4
HR =
RPE =

HORMONE II

Participant ID
Week 12
DATE:

Name:
Home:
Work:
Email:

TEMPO THRESHOLD MAX [illegible]

Session:	Date:	Type	Time	Intensity	Equipment						
							1	2	3	4	RPE
1		Warm up	20		Bike	Peak HR					
						Wattage					

Comments: 10 min warm up (get up to about max wattage, then back down); 2 min ON; 3 min OFF x 4; 10 min cool down
HR =
RPE =

Session	Date	Type	Time	Intensity	Equipment		Time	Av. HR	Wattage	RPE	Equipment
2		Recovery	40		Bike						

Comments:
HR =
RPE =

Session	Date	Type	Time	Intensity	Equipment		Time	Av. HR	Wattage	RPE	Equipment
3		Threshold	50		Bike	1					
						2					

Comments: 10 min warm up; 2 x 10 min @ Threshold; 10 min rest 10 minute cool down
HR =
RPE =

Session	Date	Type	Time	Intensity	Equipment		Time	Av. HR	Wattage	RPE	Equipment
4		Tempo Grinds	[illegible]		Anything						

Comments: 8 mins at tempo and 2 mins grinds (lower cadence (50-60 rpm), increase resistance to approx. threshold W)x 4

Appendix 8 - Study One participant data

High Fitness Group.

ID	Age	Height	Weight	BMI	SSF	VO_{2max}	F 2-OHE1	F 16α-OHE1	L 2-OHE1	L 16α-OHE1	F 2:16α-OHE1	L 2:16α - OHE1
	y	cm	Kg	m/kg^2	mm	ml/kg/min	ng/ml/mg Cr	ng/ml/mg Cr	ng/ml/mg Cr	ng/ml/mg Cr		
1	23	159.0	53.5	21.2	54.10	56.4	6.8	8.4	44.2	26.9	0.82	1.64
2	25	167.8	68.4	24.3	55.50	50.1	8.5	9.1	16.8	9.3	0.93	1.80
3	34	168.4	53.2	18.8	28.80	59.6	16.5	8.6	26.0	13.5	1.93	1.92
4	35	162.0	60.5	23.1	31.00	52.0	9.1	6.9	23.5	14.8	1.31	1.59
5	36	161.4	55.9	21.4	28.00	53.3	5.9	4.1	29.7	10.5	1.96	2.78
7	22	161.0	66.0	25.5	35.20	49.4	7.4	3.8	26.8	16.3	1.45	2.83
8	23	168.2	65.5	23.2	46.80	47.9	8.2	6.0	20.3	9.9	1.97	1.65
9	41	166.8	60.2	21.6	50.20	59.2	11.8	6.7	17.1	9.3	1.38	2.06
10	38	162.2	47.0	17.9	24.90	51.0	16.6	7.9	36.4	13.8	1.75	1.85
11	24	167.2	55.4	19.8	33.50	53.3	4.1	9.8	18.4	8.9	2.10	2.64
12	23	168.0	65.2	23.1	27.60	57.6	15.5	11.3	14.8	5.6	0.42	2.07
13	42	160.0	55.0	21.5	29.90	52.7	9.1	5.7	21.1	13.6	1.36	2.67
14	24	160.0	53.8	21.0	27.40	48.0	11.8	9.5	33.5	16.6	1.61	1.55
15	29	173.2	60.8	20.3	39.70	55.8	10.4	11.6	22.6	15.3	1.25	2.01
16	32	169.3	62.3	21.7	26.00	50.1	6.6	6.3	12.2	10.0	0.89	1.48
17	20	163.5	55.1	20.6	34.60	51.2	8.9	8.2	19.6	21.6	1.04	1.22
18	30	170.6	70.8	24.3	28.10	49.6	10.7	9.2	28.2	15.7	1.09	0.92

Legend: BMI, body mass index; SSF, sum of four skin fold (triceps, biceps, subscapular and iliac crest); VO_{2max}, maximal oxygen consumption; F, follicular phase of the menstrual cycle; L, luteal phase of the menstrual cycle; 2-OHE1, 2-hydroxyestrone; 16α-OHE1, 16α-hydroxyestrone.

Average fitness group

ID	Age y	Height cm	Weight Kg	BMI m/kg^2	SSF mm	VO$_2$ ml/kg/min	F 2:OHE1 ng/ml/mg Cr	F 16α-OHE1 ng/ml/mg Cr	L 2:OHE1 ng/ml/mg Cr	L 16α-OHE1 ng/ml/mg Cr	F 2:16α-OHE1	L 2:16α -OHE1
1	26	172.90	66.20	22.1	73.30	33.00	10.7	9.2	28.2	15.7	1.16	1.79
2	28	171.50	54.30	18.5	45.10	37.30	10.0	5.4	17.5	15.2	1.87	1.15
3	24	157.80	63.30	25.4	73.30	36.00	8.3	13.1	14.5	19.9	.64	.73
4	33	170.00	63.90	22.1	35.00	39.80	16.5	4.9	20.9	9.5	3.34	2.20
5	27	160.50	59.60	23.1	48.80	30.00	9.8	11.9	19.2	24.0	.83	.80
6	23	162.90	60.80	22.9	53.30	37.10	10.6	9.8	36.6	25.0	1.08	1.47
7	38	173.60	71.80	23.8	43.00	41.00	7.8	8.7	16.5	16.8	.90	.98
8	41	168.00	57.30	20.3	43.30	37.30	4.4	4.8	29.3	16.0	.91	1.83
12	33	164.20	61.50	22.8	66.70	33.10	7.7	5.4	21.1	9.7	1.44	2.16
14	27	160.00	65.40	25.5	54.30	37.90	7.6	6.1	34.7	19.2	1.25	1.80
15	30	157.90	45.30	18.2	35.80	31.70	10.5	4.8	24.7	7.9	2.18	3.11
17	21	169.50	63.30	22.0	49.40	29.10	9.1	9.0	12.9	12.6	1.01	1.02
18	25	171.70	54.00	18.3	56.50	35.10	8.5	6.2	15.0	8.5	1.37	1.77

Legend: BMI, body mass index; SSF, sum of four skin fold (triceps, biceps, subscapular and iliac crest); VO$_{2max}$, maximal oxygen consumption; F, follicular phase of the menstrual cycle; L, luteal phase of the menstrual cycle; 2-OHE1, 2-hydroxyestrone; 16α-OHE1, 16α-hydroxyestrone.

Appendix 9 – Study Two participant data

Anthropometics

ID	Age (y)	Height (cm)	Body mass (kg)			BMI (kg/m^2)			Body fat (%)	
			Pre	Mid	Post	Pre	Mid	Post	Pre	Post
1	22	177.0	61.30	61.9	61.2	19.6	19.8	19.5	32.5	30.7
2	34	163.0	63.8	62.9	61.7	24.0	23.7	23.2	35.6	34.3
3	29	159.0	60.1	59.5	58.9	23.8	23.5	23.3	35.1	33.2
4	21	168.0	71.9	66.2	66.3	25.5	23.5	23.5	38.5	34.0
6	20	163.0	69.5	67.2	66.7	26.2	25.3	25.1	40.4	39.5
7	21	174.0	71.1	73.7	74.0	23.5	24.3	24.4	31.8	35.8
9	35	168.0	69.6	68.7	69.3	24.7	24.3	24.6	28.4	29.0
10	28	164.5	71.5	73.2	73.2	26.3	27.1	27.1	33.5	33.8
11	35	158.0	57.0	59.1	59.4	22.8	23.7	23.8	35.7	37.1
13	22	172.5	84.9	87.5	87.6	28.5	29.4	29.4	37.3	38.8
14	35	167.5	60.9	61.3	61.0	21.7	21.8	21.7	34.8	34.0
15	26	160.0	54.7	53.9	53.0	21.4	21.1	20.7	33.6	32.3
16	27	173.0	61.1	62.2	62.2	20.4	20.8	20.8	28.3	25.2
18	30	169.0	77.7	78.5	78.1	27.2	27.5	27.3	46.4	45.9
19	26	179.0	77.5	76.3	75.5	24.2	23.8	23.6	27.2	27.4
20	23	167.0	50.0	50.8	50.7	17.9	18.2	18.2	20.5	21.9
21	19	172.0	52.8	54.0	54.5	17.8	18.3	18.4	25.7	24.9
22	25	165.5	74.0	76.0	76.0	27.0	27.7	27.7	43.0	43.7
23	35	172.0	69.1	71.8	72.2	23.4	24.3	24.4	38.5	38.3
24	23	166.0	52.9	54.3	53.6	19.2	19.7	19.5	32.2	32.7
26	20	167.0	57.8	57.5	56.8	20.7	20.6	20.4	22.9	23.7
28	23	158.0	52.5	52.7	53.0	21.0	21.1	21.2	22.4	24.2
29	24	170.5	75.0	NA	76.1	25.8	NA.	26.2	41.7	43.6
31	22	171.0	68.2	NA	66.5	23.3	NA.	22.7	36.0	35.6
32	21	177.0	71.2	72.5	69.8	22.7	22.7	22.4	30.0	27.5

Anthropometrics cont'd

ID	Age (y)	Height (cm)	Body mass (kg)			BMI (kg/m^2)			Body fat (%)	
			Pre	Mid	Post	Pre	Mid	Post	Pre	Mid
35	23	165.5	59.7	59.7	61.6	21.8	21.8	22.5	28.4	30.5
33	24	168.0	66.5	65.4	65.0	23.6	23.2	23.0	35.6	35.3
34	28	167.0	63.3	62.9	61.8	22.7	22.6	22.2	32.8	31.1
36	26	165.5	61.8	62.8	62.2	22.6	22.9	22.7	32.1	32.2
37	26	169.0	66.2	65.2	66.2	23.2	22.8	23.2	36.8	36.7
38	21	172.0	58.8	60.1	59.7	19.9	20.3	20.2	23.0	26.4
40	28	168.0	59.4	NA.	58.9	21.0	NA.	20.9	35.3	34.5

Legend: BMI, body mass index; Pre, baseline measure; Mid, mid-point; Post, postintervention

Notes: Body fat measured by DEXA

Aerobic Fitness

ID	VO_{2max} (ml/kg/min)			VO_{2max} (L/min)			Maximal power output (watts)		
	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post
1	37.0	40.2	37.4	2.27	2.49	2.29	186	203	221
2	38.0	36.5	41.6	2.43	2.24	2.56	205	213	233
3	30.7	35.4	37.0	1.84	2.11	2.18	150	205	200
4	37.7	44.5	40.5	2.71	2.94	2.69	212	240	250
6	31.4	35.5	32.5	2.18	2.38	2.17	181	203	233
7	34.1	NA.	28.9	2.47	NA.	2.14	191	NA.	172
9	35.3	NA.	35.2	2.45	NA.	2.44	223	NA.	233
10	33.0	NA.	30.2	2.36	NA.	2.21	233	NA.	206
11	28.6	NA.	27.8	1.63	NA.	1.65	113	NA.	144
13	27.3	NA.	25.6	2.32	NA.	2.24	188	NA.	220
14	27.7	NA.	26.3	1.68	NA.	1.61	142	NA.	147
15	28.4	32.8	34.7	1.55	1.77	1.84	140	173	173
16	38.3	37.9	40.3	2.34	2.36	2.49	220	226	247
18	26.0	27.2	31.2	2.02	2.14	2.43	163	206	218
19	37.5	NA.	34.8	2.90	NA.	2.63	238	NA.	242
20	39.4	NA.	38.6	1.97	NA.	1.96	178	NA.	184
21	34.4	NA.	41.0	1.82	NA.	2.23	178	NA.	226
22	24.9	26.4	30.7	1.84	2.01	2.33	178	184	209
23	30.7	33.2	37.0	2.12	2.39	2.68	192	213	222
24	31.6	NA.	32.5	1.67	NA.	1.74	144	NA.	147
26	38.9	NA.	39.5	2.24	NA.	2.20	211	NA.	192
28	34.4	35.3	42.8	1.81	1.86	2.27	178	184	212
29	28.3	NA.	28.8	2.12	NA.	2.19	173	NA.	178
31	33.9	NA.	34.4	2.31	NA.	2.29	206	NA.	181
32	33.8	32.6	34.6	2.40	2.31	2.41	200	233	216
33	30.8	NA.	32.0	2.05	NA.	2.08	204	NA.	192
34	26.5	29.0	32.1	1.68	1.83	1.99	140	181	188

Aerobic Fitness cont'd

ID	VO_{2max} (ml/kg/min)			VO_{2max} (L/min)			Maximal power output (watts)		
	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post
35	32.6	NA.	32.2	1.94	NA.	1.99	184	NA.	195
36	35.8	40.5	46.1	2.21	2.54	2.87	184	199	224
37	32.5	34.9	35.5	2.15	2.27	2.35	215	233	233
38	37.1	38.6	40.8	2.18	2.32	2.43	206	212	247
40	32.1	NA.	29.8	1.90	NA.	1.76	178	NA.	162

Legend: VO_{2max}, maximal oxygen consumption; Pre, baseline measure; Mid, mid-point; Post, postintervention

Estrogen Metabolites

ID	Cycle 1			Cycle 2			Cycle 3			Cycle 4		
	2:OHE1 Ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1	2:OHE1 ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1	2:OHE1 ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1	2:OHE1 ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1
1	32.08	15.47	2.07	40.00	14.62	2.74	31.49	13.24	2.38	56.12	15.10	3.72
2	31.42	15.30	2.05	24.02	7.11	3.38	22.65	9.72	2.33	26.15	12.49	2.09
3	43.67	22.04	1.98	41.26	14.59	2.83	47.41	23.01	2.06	41.63	23.45	1.77
4	22.82	36.13	0.63	23.94	42.52	0.56	48.21	55.37	0.87	32.78	60.48	0.54
6	15.73	17.01	0.92	7.16	10.91	0.66	49.11	63.46	0.77	49.11	63.46	0.77
7	32.43	10.87	2.98	24.31	7.68	3.16	23.15	6.74	3.43	43.47	19.73	2.20
9	12.20	5.39	2.26	19.52	6.70	2.91	14.81	6.40	2.32	33.02	12.27	2.69
10	4.49	7.70	0.58	6.45	6.01	1.07	11.60	7.78	1.49	7.46	5.79	1.29
11	11.88	10.79	1.10	29.30	22.01	1.33	14.53	9.90	1.47	28.30	22.50	1.26
13	15.22	13.07	1.16	16.42	19.55	0.84	12.26	16.40	0.75	15.70	18.81	0.83
14	11.83	9.98	1.19	17.44	15.24	1.14	13.63	11.57	1.18	22.83	12.01	1.90
15	26.77	9.94	2.69	12.49	6.97	1.79	27.07	12.84	2.11	26.07	11.73	2.22
16	5.19	5.09	1.02	17.74	14.09	1.26	16.08	13.77	1.17	14.11	7.90	1.79
18	7.12	11.01	0.65	10.17	33.52	0.30	4.46	14.82	0.30	3.45	12.46	0.28
19	38.78	18.29	2.12	29.00	17.87	1.62	16.73	15.39	1.09	28.43	13.89	2.05
20	29.12	14.50	2.01	39.06	14.24	2.74	48.85	25.09	1.95	68.35	30.20	2.26
21	7.75	5.53	1.40	7.75	5.53	1.40	7.75	5.53	1.40	7.75	5.53	1.40
22	137.94	77.71	1.78	25.08	13.34	1.88	35.09	13.24	2.65	54.29	29.17	1.86
23	61.63	23.63	2.61	44.33	19.37	2.29	45.70	16.07	2.84	54.26	28.01	1.94
24	37.30	25.47	1.46	66.49	35.05	1.90	29.76	20.21	1.47	29.76	20.21	1.47
26	62.30	13.84	4.50	30.33	6.37	4.76	28.09	12.05	2.33	28.09	12.05	2.33
28	23.88	21.72	1.10	32.14	29.63	1.08	14.18	13.70	1.03	45.41	31.81	1.43
29	64.98	23.01	2.82	20.78	9.84	2.11	20.39	18.84	1.08	17.25	7.57	2.28
31	40.79	15.94	2.56	43.50	39.23	1.11	53.14	54.42	0.98	51.30	33.64	1.53
32	29.97	16.20	1.85	34.35	18.53	1.85	41.13	18.06	2.28	23.85	15.95	1.50

Estrogen Metabolites, cont'd

ID	Cycle 1			Cycle 2			Cycle 3			Cycle 4		
	2:OHE1 ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1	2:OHE1 ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1	2:OHE1 ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1	2:OHE1 ng/ml/mg Cr	16α-OHE1 ng/ml/mg Cr	2:16-OHE1
33	2.41	3.84	0.63	41.14	13.59	3.03	26.03	13.50	1.96	19.82	8.96	2.21
34	21.32	15.20	1.40	26.88	19.35	1.39	26.67	14.96	1.78	24.58	13.20	1.86
35	40.66	16.68	2.44	40.66	16.68	2.44	29.71	20.08	1.48	22.39	27.87	0.80
36	21.13	6.79	3.11	16.08	11.91	1.35	14.64	9.23	1.59	17.35	11.71	1.48
37	23.03	13.94	1.65	42.27	19.61	2.16	19.40	10.93	1.77	26.14	13.36	1.96
38	27.22	8.19	3.32	25.39	10.89	2.33	18.93	8.99	2.11	33.88	15.05	2.25
40	9.27	15.20	0.61	9.27	15.20	0.61	9.27	15.20	0.61	11.87	11.95	0.99

Legend: 2-OHE1, 2-hydroxyestone; 16α-OHE1, 16α-hydroxyestone.